Roche Molecular Biochemicals

LightCycler Operator's Manual
Version 3.0

May 1999

Prologue

The LightCycler Instrument of Roche Molecular Biochemicals, Mannheim, Germany, is a thermocycler for the rapid analysis of PCR applications.

LightCycler technology is the most innovative and rapid possibility for carrying out and simultaneously evaluating PCR experiments. 30 amplification steps including analysis of results can be carried out in less than 30 minutes. Fluorimetric analysis of the PCR products formed is performed as real-time measurement either continuously or at a specifically defined time during each PCR cycle. The analyses can be monitored online by the LightCycler's user-friendly software, i.e. directly during the reaction.

This Operator's Manual contains all the necessary technical information required to place the LightCycler instrument in operation and to carry out practical analyses. It contains background information on some of the applications and enables the quick and safe optimization of individual applications.

Symbols and Notes

Symbols and Headings

The following symbols and headings are used in this manual:

Symbol	Heading	Description
	WARNING	This heading and symbol are used to indicate that non-compliance with instructions or procedures may lead to injury or even death.
	CAUTION	This heading and symbol are used to indicate that non-compliance with instructions or procedures may cause damage to the instrument.
	NOTE	This heading is used to bring your attention to topics of importance

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Chapter A
Technical Aspects of the LightCycler Instrument

1. Technical Aspects of the LightCycler Instrument

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1.2 LightCycler System

Note

Using the system improperly may compromise the integrity of the results, result in poor performance, or even permanently damage the equipment.

Components of the LightCycler System

The components of the LightCycler System are listed in the following table. Please note that these components may vary from country to country.

Component	Description
System component 1	• LightCycler Instrument • Sample Carousel (for Ø 1.5 mm capillary)[1] premounted in LightCycler Instrument
System component 2	• LightCycler Capillaries (96 capillaries and stoppers/box)[1] • 32 LightCycler Centrifuge Adapters in an aluminum cooling block[1] • 1 LightCycler Operator's Manual • 1 LightCycler Software Package[1] • Cable to connect LightCycler Instrument to the computer • Power Cord (German) • Power Cord (U.S.) • Mouse Pad
Hardware	• PC with Pentium processor[2] • 64 MB SDRAM (minimum configuration) • 24x CD-ROM drive • Keyboard and PS/2 mouse • Internal Iomega ZIP drive
Operating system	• Windows NT 4.0, including Service Pack III
Monitor	• 17" monitor[2]
Printer	• Hewlett-Packard Color Inkjet Printer[2]

[1]Also available as single components:

LightCycler Sample Carousel	Cat. no. 1 909 282 (1 piece)
LightCycler Capillaries	Cat. no. 1 909 339 (1 set, 768 capillaries)
LightCycler Centrifuge Adapters	Cat. no. 1 909 312 (1 Set)
LightCycler Software Package	Cat. no. 1 909 304

[2] Computer workstation configuration may vary from country to country and may change due to component availability and technical advances. To confirm exact computer configuration, please contact your local Roche Molecular Biochemicals representative.

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Marks of Conformity

The LightCycler has been manufactured according to EN 61010-1 (Safety regulations for Measuring, Control and Laboratory Instruments; Part 1: General Requirements [IEC 1010-1 + A1: 1992, modified]) and has been checked in accordance with all relevant safety standards prior to leaving the factory.

The instrument has been approved for use by recognized testing institutions.

This is confirmed by the following test/conformity symbols:

Acronym	Test Symbol	Testing Institution
GS		Certified by TÜV Product Service
CE		The instrument conforms to current directives as issued by the European Union
UL		Certified by Underwriters Laboratories Inc.
CUL		Certified by Underwriters Laboratories for Canada – a testing facility recognized by the Standards Council of Canada (SSC)

Equipment to be connected must fulfill the standards set by IEC 950 (Information security in technical equipment, including electronic business machines).

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Classification

The LightCycler Instrument is classified as:

- ISM instrument (Industrial Scientific Medical Device), medium-sized, for industrial, laboratory and domestic use.
- Designed for stationary operation.
- Intended for worldwide use.
- Intended for evaluating preprocessed biological material.

Note on Use with Infectious Material

- The instrument may not be used to analyze infectious materials unless additional safety measures to ensure safe sample handling (e.g., placing the instrument in a laminar flow biological safety cabinet) are taken beforehand.

1.3 Technical Data

1.3.1 Specifications of the LightCycler

General Data

Dimensions	Length: 45 cm Width: 30 cm Height: 40 cm
Weight	19.2 kg
Power supply	110–240 V, +/–10%, 47–63 Hz
Wattage	max. 800 W
Maximum current	Europe: 4 A (220 V) US: 8 A (110 V)
Noise in accordance with DIN 43635	<60 dBA
Heat emission, including PC, monitor and printer	max. 850 W
Safety symbols	CE, GS, UL, CSA or CUL

Environmental Parameters

Temperatures allowed during operation	15° to 35°C
Temperatures required to maintain specifications during operation	18° to 30°C
Temperatures allowed during transportation/storage/packaging	–20° to +60°C
Relative humidity	20 to 80%, no condensation
Altitude/pressure	0 to 2000 m above sea level, 1030 to 850 hp

Samples

Number of samples per run	32
Sample volume	10 to 20 µl

1.3.2 Specifications for Applications

Temperature for PCR

Temperature range	40° to 98°C
Accuracy of “capillary temperature” at thermal equilibrium	±0.4°C
Accuracy of “displayed temperature” with respect to capillary temperature at thermal equilibrium	±0.4°C
Capillary Heating Rates	***Volume : Heating Time Required***
Heating rate 40° to 95°C (non-linear)	10 µl: ≤13 s 20 µl: ≤15 s
Heating rate 50° to 72°C (non-linear)	10 µl: ≤6 s 20 µl: ≤8 s
Heating rate 72° to 95°C (non-linear)	10 µl: ≤6 s 20 µl: ≤8 s
Capillary Cooling Times	***Volume : Cooling Time Required***
Cooling rate 95° to 40°C (non-linear)	10 µl: ≤23 s 20 µl: ≤24 s
Cooling rate 95° to 60°C (non-linear)	10 µl: ≤7 s 20 µl: ≤7 s
Temperature Tolerances, Short Term	
Precision of capillary temperature over all capillary positions when measured for 30 s at 95°C	±1.5°C
Precision of capillary temperature over all capillary positions when measured for 30 s at 70°C	±1.0°C
Precision of capillary temperature over all capillary positions when measured for 30 s at 45°C	±0.5°C
Temperature Difference, Melting Curves	
Systematic difference between sensor temperature and capillary temperatures when chamber is heated from 50°C to 95°C at a rate of 0.2°C/s	<0.1°C

1.3.3 Specifications for the Detectors

Excitation

Type	LED
Median wavelength	470 nm
Wattage at 470 to 490 nm	0.1 mW

Filter

Detector 1	Bandpass 530 nm, HBW 20 nm, dichroic
Detector 2	Bandpass 640 nm, HBW 20 nm, dichroic
Detector 3	Bandpass 710 nm, HBW 40 nm, dichroic

Detector

Type	Photohybrid
Sensitivity at 530 nm, 20 µl sample volume	10 fM fluorescein
Resolution	12 bit
Range of detection sensitivity	Adjustable by a factor of 1 to 256

Typical Time

Signal acquisition time for 32 capillaries	≈ 5.0 s

1.3.4 Temperature Control

Visual Display of Temperature Profiles

The LightCycler software graphically displays the current temperature in the thermal chamber. These graphs appear under **Temperature History** on the **Running Screen** (see below).
The graphs show “overshoots” and “undershoots” for each temperature transition (see arrows below).

Continued on next page

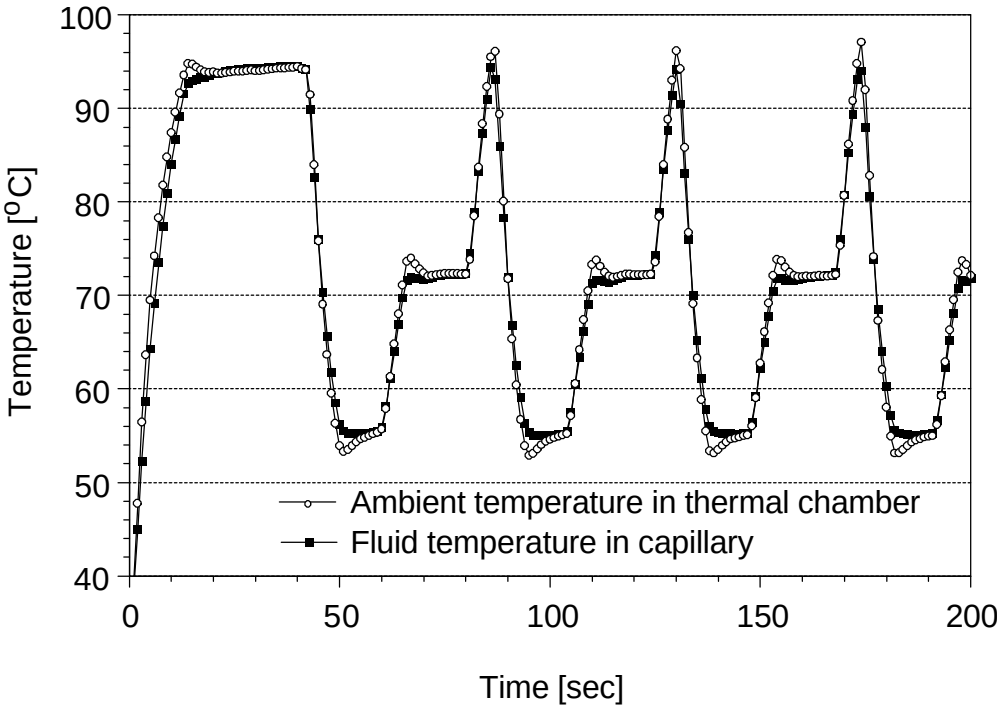
1.3.4 Temperature Control, Continued

Autocorrection

Autocorrection compensates for physical differences in heat capacity in air and water. This specific autocorrection ensures that every programmed temperature is adjusted **within** the sample capillaries during thermal cycling.

Effectiveness of Autocorrection

The graph below shows the changes in temperature in both the thermal chamber and a sample capillary during thermal cycling. Differences at any given time between the temperature in the two locations are shown as spaces between the two temperature curves.



Conclusion: This graph demonstrates that:

- The temperature profile shown under **Temperature History** on the **Running Screen** exactly corresponds to the capillary temperature, and
- The fluid temperature in the capillaries coincides with the programmed profile.

1.4 Safety Precautions for the LightCycler

General Safety Precautions

Always observe these precautions when using the LightCycler Instrument:

- Follow all safety instructions printed on, or attached to, the analytical instruments.
- Observe all general safety precautions which apply to electrical instruments.
- Never touch switches or power cord with wet hands.
- Do not open the housing of the LightCycler.
  Note: Only authorized service personnel should perform service or repairs required for this unit.
- Do not open the LightCycler thermal chamber during operation.
- The chamber lid and the sample rotor are hot while the instrument is operating.
  Note: The corresponding symbol is attached to the front side of the LightCycler.
- Always wear safety goggles and gloves when dealing with toxic, caustic, or infectious materials .
- When analyzing infectious materials, use the LightCycler only in specially equipped rooms with controlled exhaust equipment, e.g., biological safety cabinets.

Safety Precautions for Centrifuge Adapters

Loading capillaries to perform fluorescence measurement with the LightCycler requires a centrifugation step in a tabletop centrifuge with specially designed adapters. A centrifuge adapter with capillary weighs approximately 6.2 grams. Always observe the following precautions during the centrifugation step:

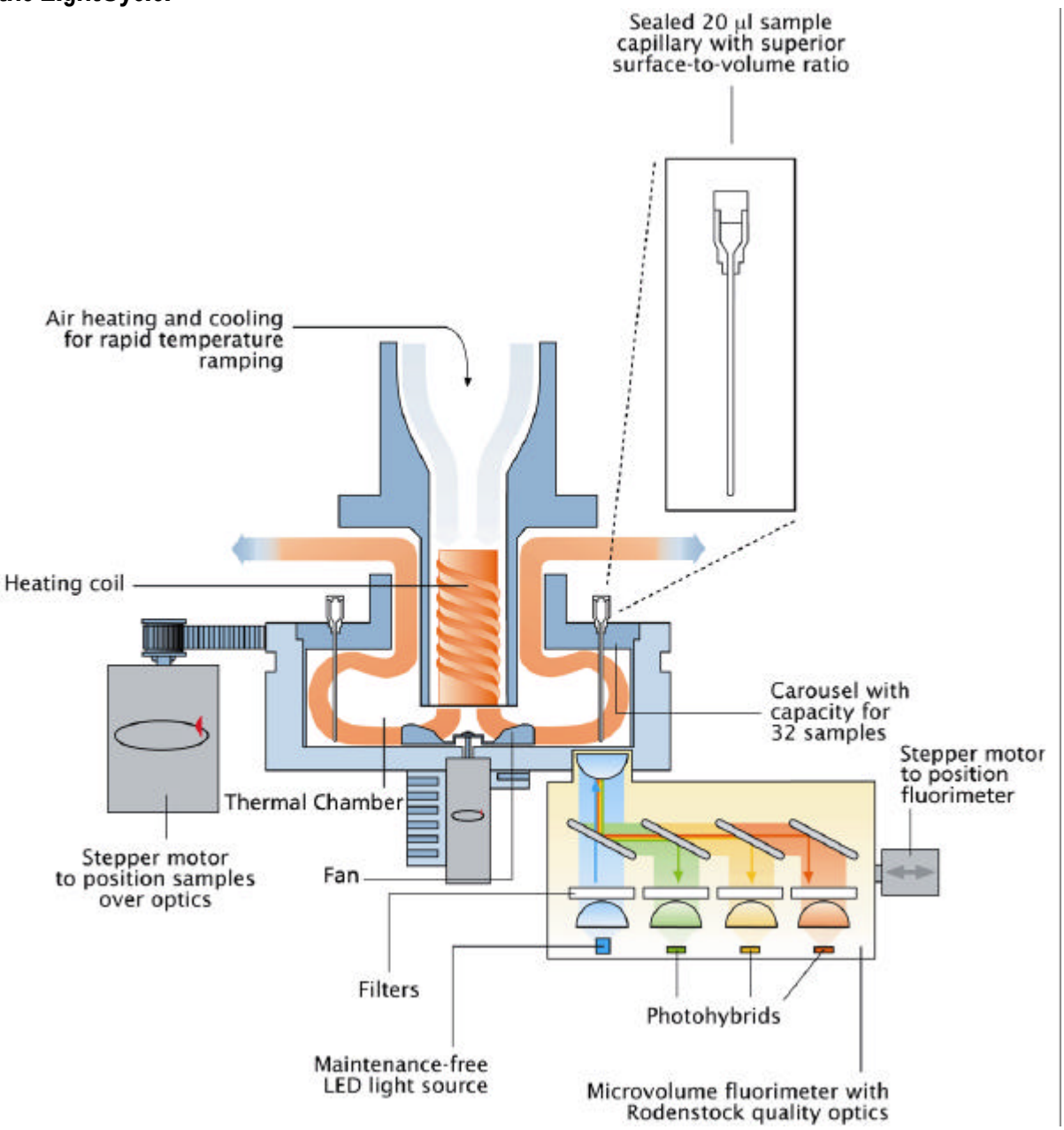
- Always distribute the adapters in the rotor so the weight in the rotor is balanced, and
- Adjust the speed of the centrifuge so the centrifugal force **does not exceed 700 x g**.

Note: For details on the maximum weight that can be processed in your centrifuge, refer to the product specification sheet for that centrifuge.

2. System Description

2.1. The LightCycler

Schematic of the LightCycler


Sealed 20 µl sample capillary with superior surface-to-volume ratio
Air heating and cooling for rapid temperature ramping
Heating coil
Carousel with capacity for 32 samples
Stepper motor to position fluorimeter
Thermal Chamber
Stepper motor to position samples over optics
Fan
Filters
Photohybrids
Maintenance-free LED light source
Microvolume fluorimeter with Rodenstock quality optics

Continued on next page

Description of the Instrument

The LightCycler basically consists of an upper unit and a lower unit. The upper unit contains the heating coil. The lower unit contains the thermal chamber, fluorimeter, drive units, electronic boards and power supply. The various elements are mounted on a 10-mm cast aluminum base plate. This guarantees stability, especially for the thermal chamber and fluorimeter.
Hot or ambient temperature air, introduced into the thermal chamber, regulates the temperature of the sample capillaries. A heating coil heats the air, which is then fed into the chamber by a fan. The fan ensures efficient air circulation and temperature homogeneity during the heating cycle.
During the cooling cycle, the fan operates at a higher speed to ensure adequate cooling.
During measurements, a stepper motor rotates the sample carousel to position the capillary tip precisely at the focal point of the fluorimeter optics. The fluorimeter itself is positioned radially to the maximum signal to compensate for any radial deviation of the capillary tip.

Communication between LightCycler and PC

For online display, data are transmitted to and from the PC via a serial interface.
Control is made possible by three microprocessors that are integrated into the instrument:

- Processor 1 is for communication,
- Processor 2 regulates the temperature, and
- Processor 3 controls the measurement procedures as well as the movement of the rotor and fluorimeter.

Users enter data on samples (number of samples, name, concentration, etc.) and on the experimental protocol into the PC; the PC transmits the data to the LightCycler. The PC also monitors the temperatures and fluorescence signals during the PCR run and operates all the analytical programs.

Power Supply Unit

All high-voltage components are located in the power-supply unit.
Note: Electrical safety information and test symbols apply to this unit.
To minimize interference to the detector electronics, the electronics that control the drive units are located on a separate circuit board.

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Temperature Setting of the Thermal Chamber

Temperature is controlled with hot air and air at ambient temperature. Varying the voltage supplied to the heating coil regulates the temperature. A sensor provides reference values for control purposes.
During the heating phase, the fan in the thermal chamber operates at low speeds to ensure homogenous distribution of temperature.
During the cooling phase, the fan operates at higher speeds so that the capillaries and the heating coil can be cooled efficiently.
Two sensors are integrated into the LightCycler to prevent unduly high temperatures:

- Sensor I is located in the thermal chamber and switches off the heat when a temperature of 125°C has been reached.
- Sensor II monitors the temperature on the aluminum base plate, and switches off the entire LightCycler to protect the electronics when the temperature exceeds 55°C.

Fluorimeter

A three-channel fluorimeter is used for detection purposes. A blue diode (LED) with maximum emission of 470 nm serves as the energy source for sample excitation. Screens are used to diffuse the light emitted by the LED to ensure uniformity. The exhaust and ventilation channels have been designed to prevent ambient light from entering directly into the thermal chamber. Fluorescence is detected at 530 nm, 640 nm, and 710 nm with the aid of photohybrids.
Note: Acquisition time for fluorescence acquisition time is 20 msec (16.6 msec in the U.S.).

Measurement of Samples

The thermal chamber and the fluorimeter are placed optimally for sample measurement. Only the sample carousel, which holds the capillary, rotates during measurement of the various samples.

Continued on next page

LightCycler Diodes

Three diodes are located at the front of the LightCycler. All of the diodes come on when the instrument is switched on. In this way, the instrument tests for proper functioning of the diodes.
During instrument operation, the diodes function as described in the table below:

Position of Diode	Label	Color of Diode	Function	Indication
Top	Power on	Green	On	Instrument is switched on
			Off	• No power • Instrument is defective
Middle	Cycling	Red	On	Instrument is running
			Flashing	Instrument is defective
Bottom	Run completed	Yellow	On	PCR run is completed
			Off	Instrument is running

2.2 PC Configuration

General

The computer workstation contains the following components:

Hardware	• PC with a Pentium processor[1] • 64 MB SDRAM (minimum configuration) • 24x CD-ROM drive • Keyboard and PS/2 mouse • Internal Iomega ZIP drive
Operating system	• Windows NT 4.0, including Service Pack III
Monitor	• 17" monitor[1]
Printer	• Hewlett-Packard Color Inkjet Printer[1]

[1]Computer workstation configuration may vary from country to country and may change due to component availability and technical advances. To confirm exact computer configuration, please contact your local Roche Molecular Biochemicals representative.

3. Installation

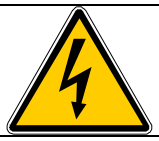
3.1 Installation Requirements

Note

- Do not place the LightCycler next to instruments that cause electromagnetic interference or have high inductance (e.g., centrifuges or mixers).
- Peripheral instruments connected to the LightCycler System must meet the IEC 950 (UL 1950) standard.
- All plugs used in the LightCycler System (PC, printer, monitor) should have the same phasing in order to prevent switch-on peaks and electronic noise generated by other instruments or by the power supply itself. We recommend using a dedicated multiple-outlet surge protector for the LightCycler System.
- Use only the supplied power lines and RS 232 connector.

Space and Power Requirements

Place the instrument in a site that can support the following instrument requirements:

<table>
<tr><td>Dimensions</td><td>The LightCycler is 30 cm wide, 45 cm long, and 40 cm high. .</td></tr>
<tr><td>Weight</td><td>Approximately 20 kg</td></tr>
<tr><td>Voltage Requirements</td><td>The LightCycler operates between 120 and 240 V (50 to 60 Hz).
<u>Note</u>: The LightCycler adjusts automatically to the available voltage when the instrument is plugged in. The user does not have to set the instrument to the correct voltage manually.
<table><tr><td>  </td><td>Do not open the LightCycler housing.</td></tr></table></td></tr>
<tr><td>Power Consumption</td><td>The LightCycler uses 800 W max.
PC and printer consume an additional 500 W (approx.).</td></tr>
</table>

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Environmental Parameters

The instrument requires the following environment for accurate operation:

Ambient temperature	15° to 35°C
Ambient temperature required to main specifications during operation	18° to 30°C
Humidity	20 to 80%, no condensation
Altitude	Sea level to 2000 m
Excess voltage	Category II
Degree of contamination	2

Storage Conditions

The LightCycler can be stored under the following conditions:

Ambient temperature	–20° to +60°C
Humidity	20 to 80%, no condensation

3.2 Installation of the LightCycler

Installation of the LightCycler Instrument

Your Roche Molecular Biochemicals representative will normally install the LightCycler Instrument at your site. Should this not be possible, follow these steps to install the instrument successfully:

Step	Action
1	Unpack the instrument.
2	Position the instrument on the workbench. Allow 10-cm spaces to the left, right and behind the instrument to ensure sufficient cooling of the electronic components. ***Note:*** The workbench should not be covered with paper.
3	Make the following electrical connections: • Connect the LightCycler to the PC using the RS 232 cable (serial interface) provided with the system. • Connect the LightCycler, PC, monitor, and printer to the same multiple-outlet distributor plug. Ensure that PC, monitor, and printer have been set to the correct voltage.

3.3 Installation of the Computer

Installation of the PC

To install the PC, do the following:

Step	Action
1	Connect mouse, keyboard, and monitor to the computer.
2	Connect the LightCycler to the computer with the RS 232 cable (serial interface) provided with the system.
3	Connect the computer, monitor, and LightCycler to the same multiple-outlet distributor plug. The computer is now ready for operation.

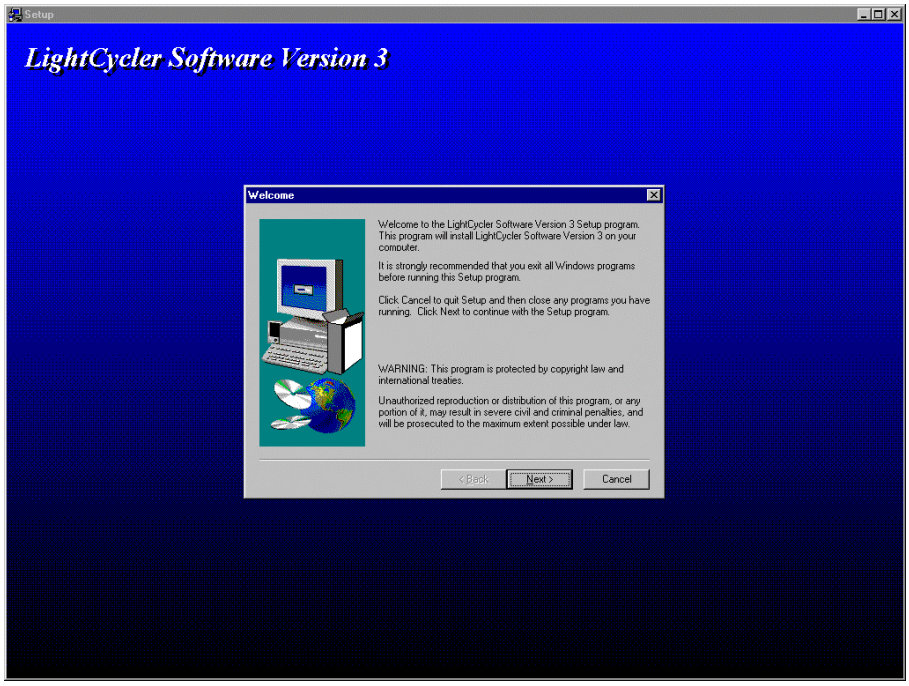
3.4 Installation of LightCycler Software Version 3

Logging In

A prerequisite for the software installation is that the operating system WIN-NT 4.0 (Service Pack 3) has been installed. For the installation of the software and the setting up of users it is recommended that you log in as administrator. All instructions are based on the English WIN NT 4.0 version. The operating system programs and folders in the German versions have different names.

Installation of LightCycler Software

Insert the CD with the LightCycler Software version 3 into the CD-ROM drive of your computer. The autorun function will automatically start the Setup program. If the autorun function is not available, the CD must be opened from the Explorer, and the 'setup' program started manually by double-clicking on it. The Setup program displays the following start screen:


Setup
LightCycler Software Version 3
Welcome
Welcome to the LightCycler Software Version 3 Setup program. This program will install LightCycler Software Version 3 on your computer.
It is strongly recommended that you exit all Windows programs before running this Setup program.
Click Cancel to quit Setup and then close any programs you have running. Click Next to continue with the Setup program.
WARNING: This program is protected by copyright law and international treaties.
Unauthorized reproduction or distribution of this program, or any portion of it, may result in severe civil and criminal penalties, and will be prosecuted to the maximum extent possible under law.
< Back
Next >
Cancel

Click the **“Next”** button to continue with the installation. Click **“Cancel”** to abort the program.

Continued on next page

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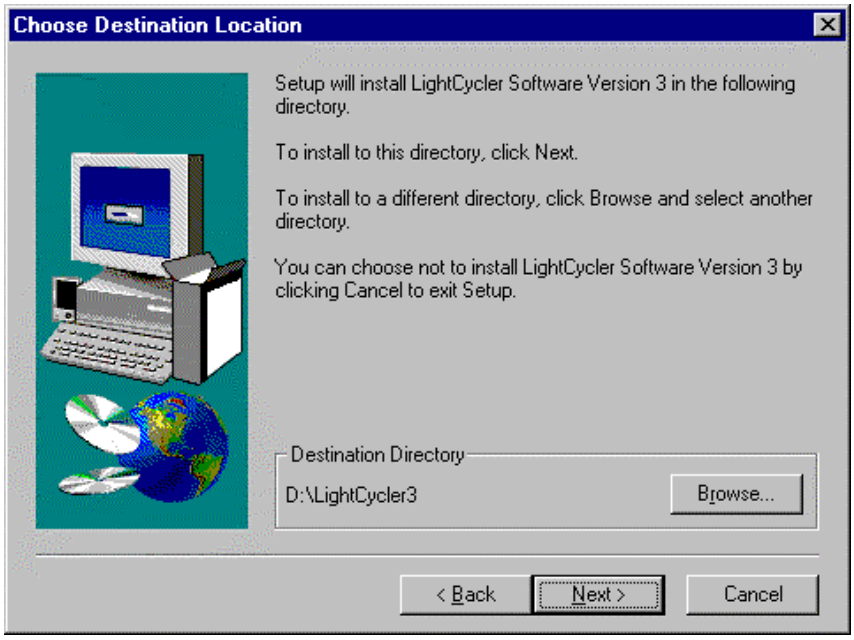
Program License Agreement
Do you accept all the terms of the preceding License Agreement? If you choose No, Setup will close. To install LightCycler Software Version 3, you must accept this agreement.
< Back
Yes
No

Here, the customer is provided with a display of the license agreement. Scroll bars can be used to view the entire text.
Choose **"Yes"** to accept the license agreement. If you choose **"No"**, the program will be exited. Clicking on **"Back"** will redisplay the start screen.

Installation Directory of LightCycler Software


Choose Destination Location
Setup will install LightCycler Software Version 3 in the following directory.
To install to this directory, click Next.
To install to a different directory, click Browse and select another directory.
You can choose not to install LightCycler Software Version 3 by clicking Cancel to exit Setup.
Destination Directory
D:\LightCycler3
Browse...
< Back
Next >
Cancel

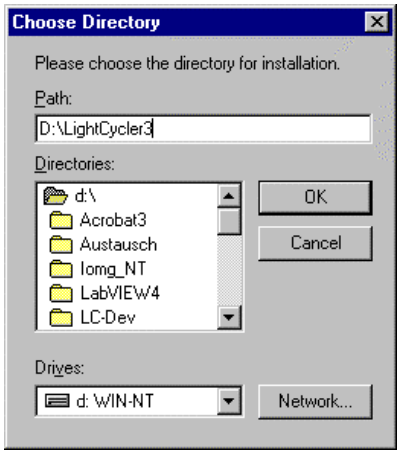
Continued on next page

Installation Directory of LightCycler Software, Continued

The software will be installed to the recommended directory **"LightCycler3"**. The disk drive corresponds to the drive to which WIN NT was installed, here: drive "D".

If **"Next"** is clicked on, the installation will be continued. If "**Cancel"** is chosen, the installation will be exited, and **"Back"** will redisplay the previous screen.

If you wish to change the disk drive or the directory, click the **"Browse"** button. A popup window will display permitting the desired path and directory to be entered.



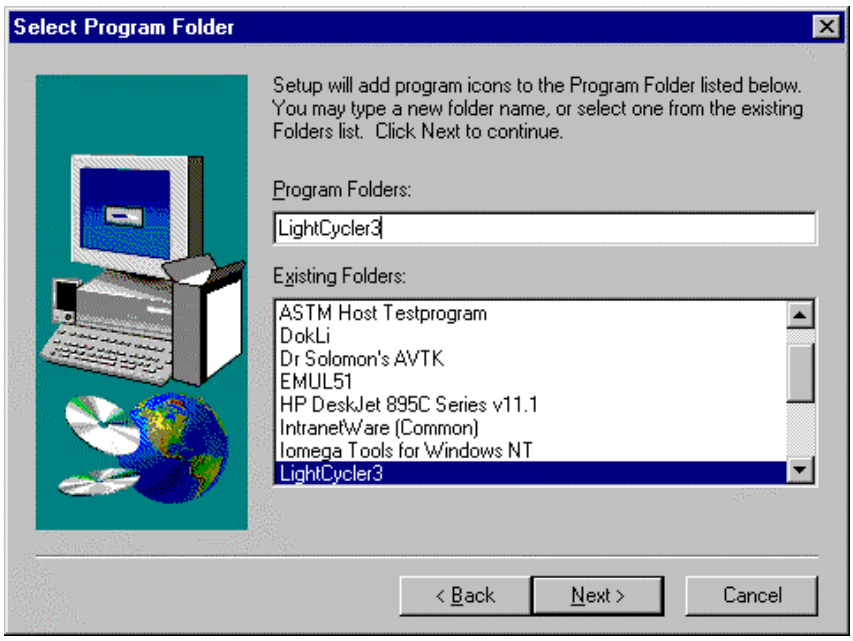
The installation to the **"LightCycler3"** directory will have no consequence for any previous installations of version V1.22 since that version was installed in the directory named **"LightCycler"**. It is therefore recommended that directory **"LightCycler3"** be used so as to avoid any conflict with respect to future versions and to be able to quickly locate the relevant versions.

Continued on next page

LightCycler Software Program Folder

The program folder contains icons to start the various LightCycler Programs. A folder named **"LightCycler3"** is set up in the Start menu under **'Programs'**. This name should be retained, if possible, to be able to locate the folder quickly.

Click **"Next"** to continue with the installation. If **"Cancel"** is chosen, the installation will be exited, and **"Back"** will redisplay the previous screen.

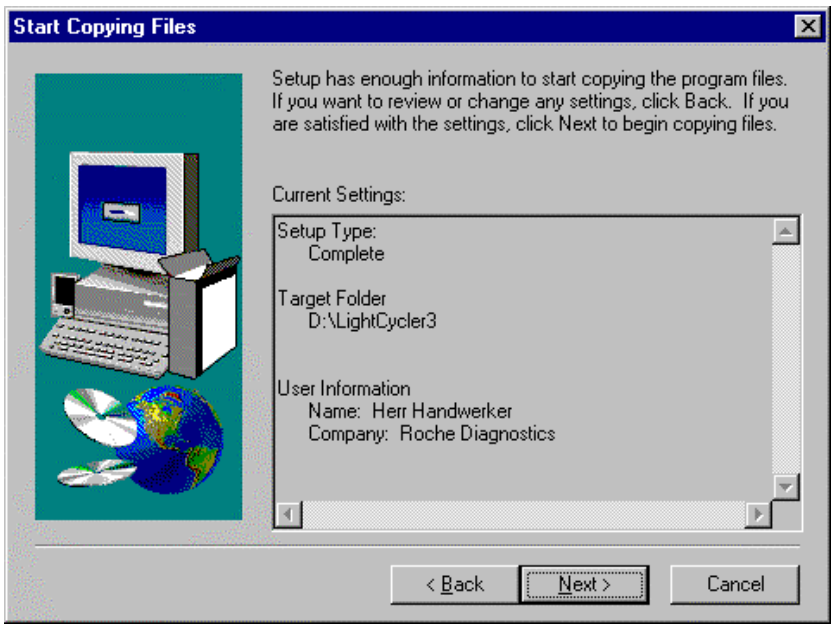

Select Program Folder
Setup will add program icons to the Program Folder listed below. You may type a new folder name, or select one from the existing Folders list. Click Next to continue.
Program Folders:
LightCycler3
Existing Folders:
ASTM Host Testprogram
DokLi
Dr Solomon's AVTK
EMUL51
HP DeskJet 895C Series v11.1
IntranetWare (Common)
Iomega Tools for Windows NT
LightCycler3
< Back
Next >
Cancel

Starting the LightCycler Software Installation

Prior to the start of the installation, a summary of the current settings is displayed. You are provided with one last chance to make changes, or to exit the installation.
Click **"Next"** to start the installation. If **"Cancel"** is chosen, the installation will be exited, and 'Back' will redisplay the screen below.

Continued on next page

Starting the LightCycler Software Installation, Continued


Start Copying Files
Setup has enough information to start copying the program files. If you want to review or change any settings, click Back. If you are satisfied with the settings, click Next to begin copying files.
Current Settings:
Setup Type:
Complete
Target Folder
D:\LightCycler3
User Information
Name: Herr Handwerker
Company: Roche Diagnostics
< Back
Next >
Cancel

LightCycler Software Installation Progress

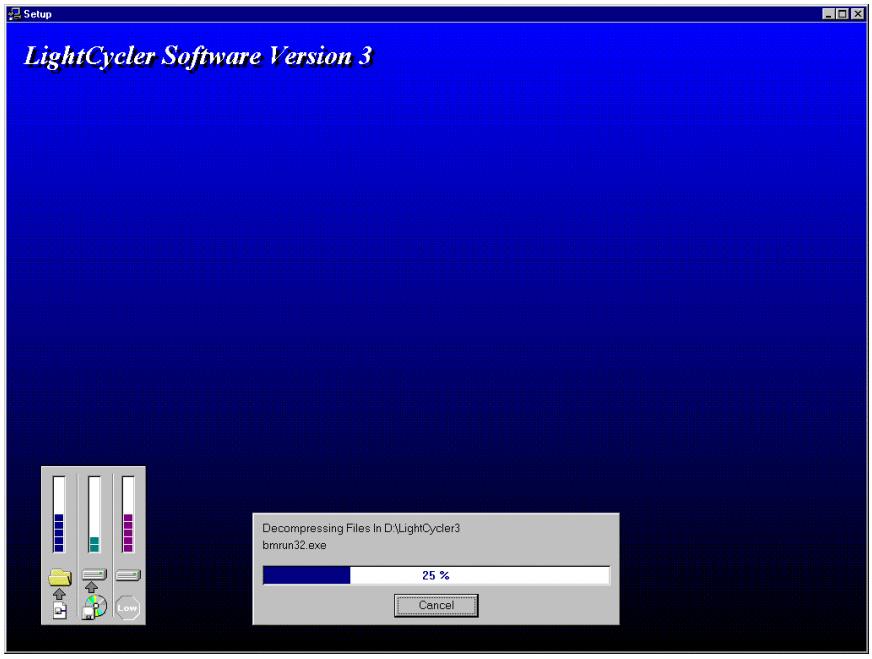
After the start of the installation, a progress bar is displayed indicating the progress of the installation. Please wait until the installation is complete and the **"Setup Complete"** screen appears.

It is possible to exit the installation by clicking **"Cancel"**. This should, however, only be done in emergencies. Any program parts that have been installed up to this point will not be removed automatically.

At the end of the installation, the program registration takes place.
A start icon for the **"Front"** program will be installed on the desktop and added to the **Start** menu. The folder **"LightCycler3"** containing the programs **"Front"**, **"BMRun"** and **"LCDA"** will be set up in the Start menu under **"Programs"**.

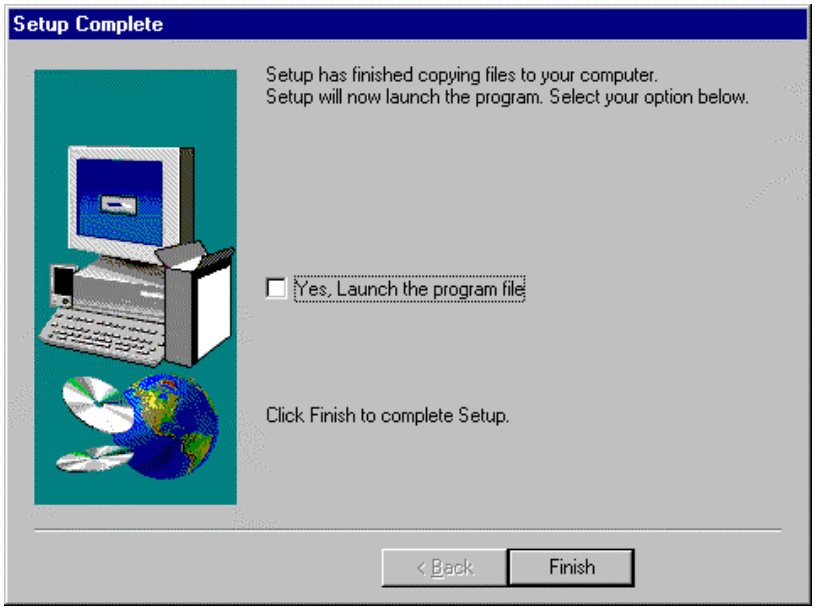
Continued on next page

LightCycler Software Installation Progress, Continued



Terminating Setup

The installation has been carried out successfully. Click **"Finish"** to complete the Setup program. If the check box **"Launch the program file"** is selected, the **LightCycler** program **"LightCycler3 Front Screen" (Front)** will start when the **"Finish"** button is clicked.


Setup Complete
Setup has finished copying files to your computer.
Setup will now launch the program. Select your option below.
Yes, Launch the program file
Click Finish to complete Setup.
< Back
Finish

Continued on next page

3.4 Installation of LightCycler Software Version 3, Continued

System File

For the LightCycler3 program to be able to run, the system file **"mfc42.dll"** is required. If an earlier version of this file is installed on your computer, it will be overwritten with the more recent version in the directory **Winnt\system32\.**

Acrobat Reader

The Acrobat Reader is required to be able to read the Help files. This program is also included on the CD. It needs only to be installed if the program has not previously been installed on the computer.

To install Acrobat-Reader, open the CD from the Explorer and start the **"ar40eng"** program by double-clicking on it in the **"Acrobat Reader"** subdirectory. The installation procedure is similar to that of the **LightCycler** Software.

Num2Dot

Num2Dot is a tool that allows use of the "comma" of the numerical pad of your keyboard to enter a decimal point. As the LightCycler Software is designed to be run under english country settings, the Software expects a "point" as a decimal point. The ability to convert the decimal "comma" on a variety of non-english keyboards to write a "point" instead significantly improves the convenience of the system.
Note: It is not required to install Num2Dot if your keyboard´s numerical pad is equipped with a "point" as decimal point.

To install Num2Dot, open the CD from the Explorer and start the install Num2Dot by double-clicking **"Setup.exe"** in the **"Num2Dot"** subdirectory. The installation procedure is similar to that of the **LightCycler** Software.

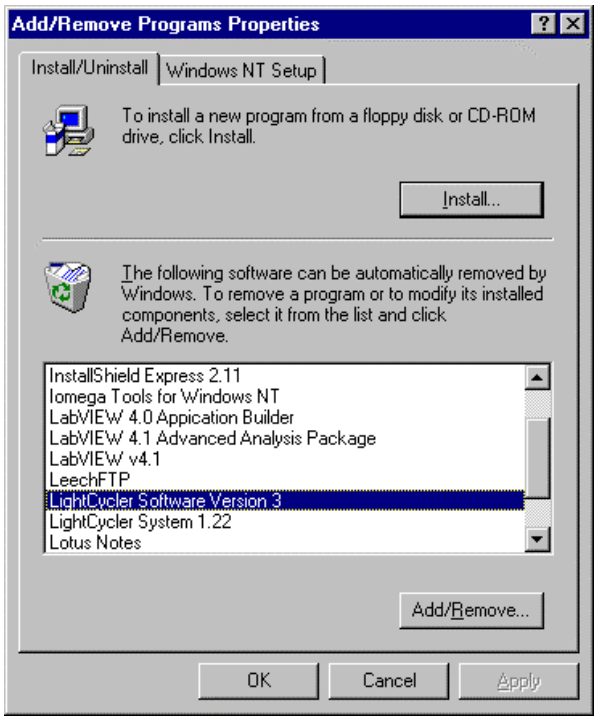
Num2Dot will automatically be started whenever you launch Windows-NT on your PC.
Verify activation of the numerical pad on your keyboard. The green light built into the "Num" key must be on.
After installation of Num2Dot, the "comma" key will be converted to a "point" key.
The Short cut command "AltGr + DecimalComma" can be used to deactivate Num2Dot, as well as to reactivate the conversion.

Continued on next page

3.4 Installation of LightCycler Software Version 3, Continued

Uninstall

To uninstall programs under WIN NT, use the **"Add/Remove Programs"** tool. This auxiliary program appears in the **Start** menu under Settings-Control Panel. A list of the programs installed is displayed. Select the program you wish to uninstall from the list and remove it by clicking the **"Add/Remove"** button.


Add/Remove Programs Properties
Install/Uninstall
Windows NT Setup
To install a new program from a floppy disk or CD-ROM drive, click Install.
Install...
The following software can be automatically removed by Windows. To remove a program or to modify its installed components, select it from the list and click Add/Remove.
InstallShield Express 2.11
Iomega Tools for Windows NT
LabVIEW 4.0 Appication Builder
LabVIEW 4.1 Advanced Analysis Package
LabVIEW v4.1
LeechFTP
LightCycler Software Version 3
LightCycler System 1.22
Lotus Notes
Add/Remove...
OK
Cancel
Apply

3.5 Dismantling the LightCycler System

Dismantling the System

To dismantle the LightCycler system, do the following:

Step	Action
1	Switch off the instrument.
2	• Disconnect the RS 232 cable and power cables. • Clean the instrument (according to the guidelines in Topic 4 of this chapter). • Decontaminate the instrument if necessary.
3	Ship instrument in its original packaging.

4. Operation and Maintenance of the LightCycler

Guidelines for Operation

- The LightCycler instrument should only be operated in locations which are protected from the weather. It may not be operated in buildings that lack facilities for regulating temperature. If necessary, additional drying agents may be used to eliminate humidity. Neither ice nor moisture should be allowed to form on the instrument.
- The instrument should be placed on an even work surface and protected from direct sunlight and outside light.
- The instrument should not be operated near dripping, spraying, splashing or running water.
- The instrument is suitable for use according to classification 3K3 in accordance with Standard EN 60721-3-3.
- The instrument should be protected from effects generated by fauna and flora.
- The instrument may be used at locations subject to noticeable or significant vibration. However, it should not be exposed to higher levels of shock waves.
- The instrument is suitable for use according to classification 3M4 in accordance with EN 60721-3-3. The instrument is able to tolerate vibrations up to classification 3M6.

Transport and Storage

When storing or transporting the instrument, do not allow it to be exposed to extreme cold (e.g., placing it in an airfreight cargo bin). Avoid temperatures lower than –25°C (since such temperatures may damage optical systems). The optical systems have open ventilation systems and should therefore be protected from dirt and humidity.

Guarantee

The terms of the guarantee are incorporated in the purchase agreement. For further details, please contact your Roche Molecular Biochemicals representative.

Continued on next page

General Maintenance

The instrument is maintenance-free.

Cleaning the Instrument

- Use 70% ethanol for disinfecting the thermal chamber.
- Clean the optical window with alcohol and an optical polishing cloth.
- Clean the housing with a mild commercial household detergent.
- Use a mild commercial household detergent for cleaning the housing.
- Do not pour fluids into the thermal chamber.

Chapter B

LightCycler Software

Version 3

1. Software

1.1. Table of Contents

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1.1 Table of Contents (continued)

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1.2. Introduction

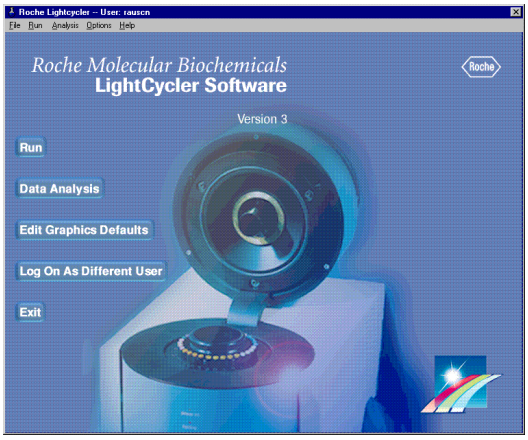
Before Beginning

Your LightCycler should have been installed and set up according to the instructions described in Section 3 of Chapter A.
During installation, the LightCycler software will automatically be loaded into the part of the hard disc that contains the Windows NT operating system.

Program Start

Prior to the start of the program, an account must be set up for each user granting him access privileges that enable him to use the computer. Such accounts must be used by the users to log on to Win NT.

If the check box **'Yes, launch the program file'** was selected at the end of the installation, the **'LightCycler3 Front Screen' (Front)** program will now start.



Log On Procedure

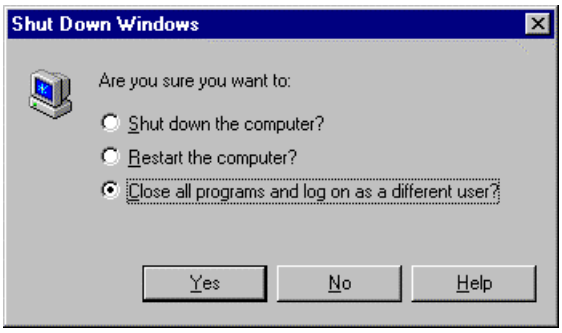
The current user may log on using the 'Log On As Different User' **button.**

The information message to the effect that all programs will be closed must be acknowledged by clicking 'OK'**. Subsequently, all programs are closed and WIN NT displays its log-on screen. The user must now log on by entering his user name and password.**
Note: **The LightCycler does not allow to log on as a different user as long as a LightCycler Run is performed to prevent loss of data by interrupting the run.**

Continued on next page

Program Start,

In the event that the check box **'Yes, launch.....'** was **not** selected at the end of the installation, the user needs to log on first. This may be accomplished in the **Start** menu using the **'Shut Down'** command.



Select the option button **'Close all Programs and log on as different user'** and click the **'Yes'** button. Windows now closes all programs and displays its log-on screen. The user must now log on by entering his user name and password.

Following the log-in procedure, the LightCycler3 program may be started. The start should always be carried out from the desktop or the Start menu by clicking the **'LightCycler3 Front Screen'** icon. Only this procedure will ensure that the user is logged on properly to the program and that the required work directories are created upon the initial startup.

Continued on next page

How to Start

Follow the steps in the table to start.

Step	Action
1	Turn on the computer by pressing the power button on the front of the computer. ***Result:*** Windows NT will automatically load.
2	Type in your User Name and Password to log into Windows NT and the LightCycler software. ***Note:*** User management requirements for the LightCycler software are set up through the Windows NT operating system.
3	Flip the switch on the back panel of the LightCycler to the **On** position.
4	Click on the **LightCycler 3 Front Screen** icon; this will bring up the **LightCycler Front Screen.** ***Note:*** Alternatively, you may use Windows NT Explorer to start each software function independently, without first entering the LightCycler Front Screen. For instance, from Windows NT Explorer, do the following: **To start from... / Click on...** LightCycler Front Screen — **Front.exe** Programming / Run Screen — **(BM) Run32.exe** LightCycler Data Analysis Front Screen — **LCDA.exe** ***Tip:*** Accessing the screens through Windows NT Explorer allows you to graphically display and evaluate data generated in previous experiments while other experimental protocols are in progress.

Important Note about the PC

The PC supplied with the LightCycler should be used exclusively with the LightCycler software. We strongly advise against installing and running additional software on this PC.

Continued on next page

1.3. The Front Screen

LightCycler Front Screen

In Version 3 of the LightCycler software, the front screen looks like this:

Introduction

When you click on the LightCycler Front Screen **icon on the Windows NT desktop, the LightCycler software is activated and the** LightCycler Front Screen **will appear on the monitor. This screen provides access to the functions of the LightCycler software via a set of menus.**

Alternatively, you may also directly access the most important instrument functions by clicking on these screen buttons:

- **Run**
- **Data Analysis**
- **Edit Graphics Defaults**
- **Log On As Different User**
- **Exit**

Note: **The functions of these buttons are explained in more detail on the pages of the next section.**

1.4. Functions of Buttons on the Front Screen

Run Button

The Run **button** starts the LightCycler Run Software which allows **you to program and execute cycle experimental protocols.**

Note: **The Front Screen** Run **menu may be used for the same purpose.**

Data Analysis Button

The Data Analysis button provides access to the LightCycler Data Analysis (LCDA) software.

Note: **The Front Screen** Analysis **menu may be used for the same purpose.**

Edit Graphics Defaults Button

The Edit Graphics Defaults **button allows a user to change the colors and line styles used in the data display graphs (see Section 1.5 of this chapter).**

Note: **The Front Screen** Options **menu may be used for the same purpose.**

Log On As Different User Button

Each user only has access to a unique set of files. To access files belonging to a different user, click on the Log On As Different User **button. This allows you to exit all LightCycler programs and reactivate the software with a different user name and password (as prompted by the system).**

Note: **The LightCycler does not allow to log on as a different user as long as a LightCycler Run is performed to prevent loss of data by interrupting the run.**

The Front Screen Options **menu may be used for the same purpose.**

Exit Button

The Exit **button allows you to quit the LightCycler program.**

Note:

- You may also quit the program by closing the Front Screen window. The **Exit** function is also part of the Front Screen **File** menu.
- After exiting the software, you still must turn off the LightCycler instrument separately.

Help Menu

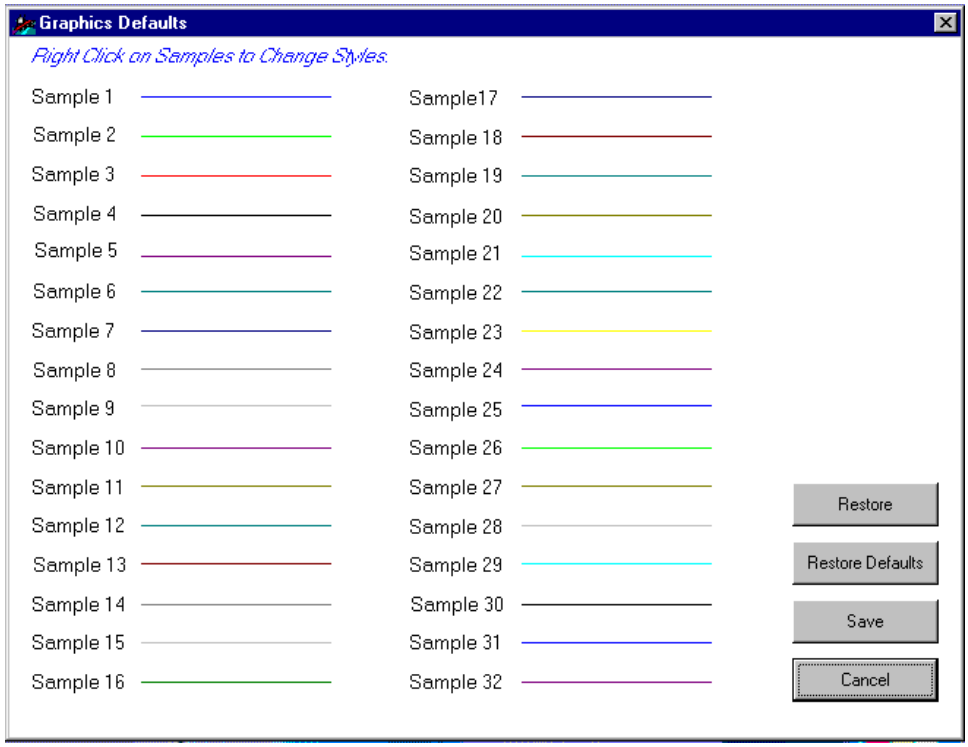
The Operator Manual can be called by Acrobat Reader.

Note: **Acrobat reader 4.0 is provided as part of the Software CD.**

1.5. Edit Graphics Defaults Window

Edit Graphics Defaults Window

The Edit Graphics Defaults button allows a user to change the color and style of the line used to represent each sample in LightCycler data displays. When you click on the button, the Edit Graphics Defaults window will appear (as shown below):



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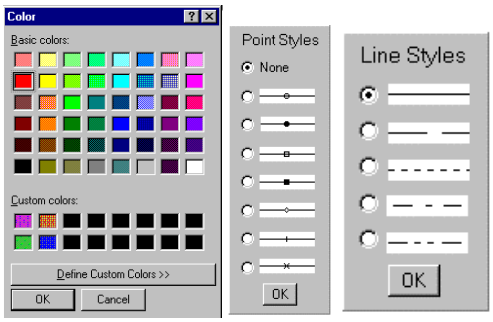
1.5. Edit Graphics Defaults Window, Continued

Changing the Line Display

After entering the Edit Graphics Defaults **window, change the appearance of the graph lines as follows:**
Caution: **Changes made to graphics defaults will not be accepted if the** LightCycler Data Analysis (LCDA) **software is active. Before changing the graphics defaults, first close** LCDA**. Then, edit and save your graphic defaults and reload** LCDA**. The changes are then accepted by** LCDA**.**
User specific default settings are stored in a file „defaults.ini" in the user directory. You may store your settings with another user by copying your user default.ini file into the other user directory.

Step	Action
1	**Right-Click on the sample number you wish to change.** *Result:* **A pop-up menu will appear. The menu contains the following graphics options:** Color, Point Style and Line Style**.**
2	**From the menu, click on the display option you wish to change.** *Result:* **One of the** Graphics Default Options **windows (pictured below) will appear.**
3	**Click on the option you prefer and then click the** OK **button.** ***Result:*** **The Graphics Default Options window will close.** *Note:* **Changes made to the graphic defaults of one user do not affect the graphics defaults of any other user.**
4	**From the** Edit Graphics Defaults **window, click on the** Save **button to save your new graphics defaults.** *Note:* **After saving them, you may always return to your display options (defined in Steps 1–4) at any time by clicking on the** Restore **button (in the** Edit Graphics Defaults **window).**
5	**If you do not like the appearance of the sample lines in the data display, you may open the** Edit Graphics Defaults **window and do one of the following:** • Click on the **Restore Defaults** button to return all options to the system default settings. • Repeat Steps 1–4 above and choose different options.

Graphics Default Options Windows: Color, Point Styles and Line Styles



2. Programming a Run

2.1. Programming Screen

Introduction

The descriptions given in the following pages explain how to use the Programming Screen **for:**

- **Defining the parameters of a PCR protocol,**
- **Starting a PCR run, or**
- **Viewing online a PCR experiment that is currently in progress.**

Activation of Programming Screen

To set up a new experimental protocol or to order a new run with a protocol that has already been defined and stored, you must first activate the Run **function from the** LightCycler Front Screen. **Follow the steps below to activate the** Run **function and open the** Programming Screen**:**

Step	Action
1	Click the **Run** button on the **LightCycler Front Screen**. ***Result:*** A data-entry screen opens. This screen will henceforth be referred to as **Programming Screen**.
2	If you have not already switched on the LightCycler instrument**,** the software will prompt you to do so. ***Note:*** It is possible to ignore this message, by simply clicking **OK**. This will allow you to become familiar with the LightCycler software without running an experiment.

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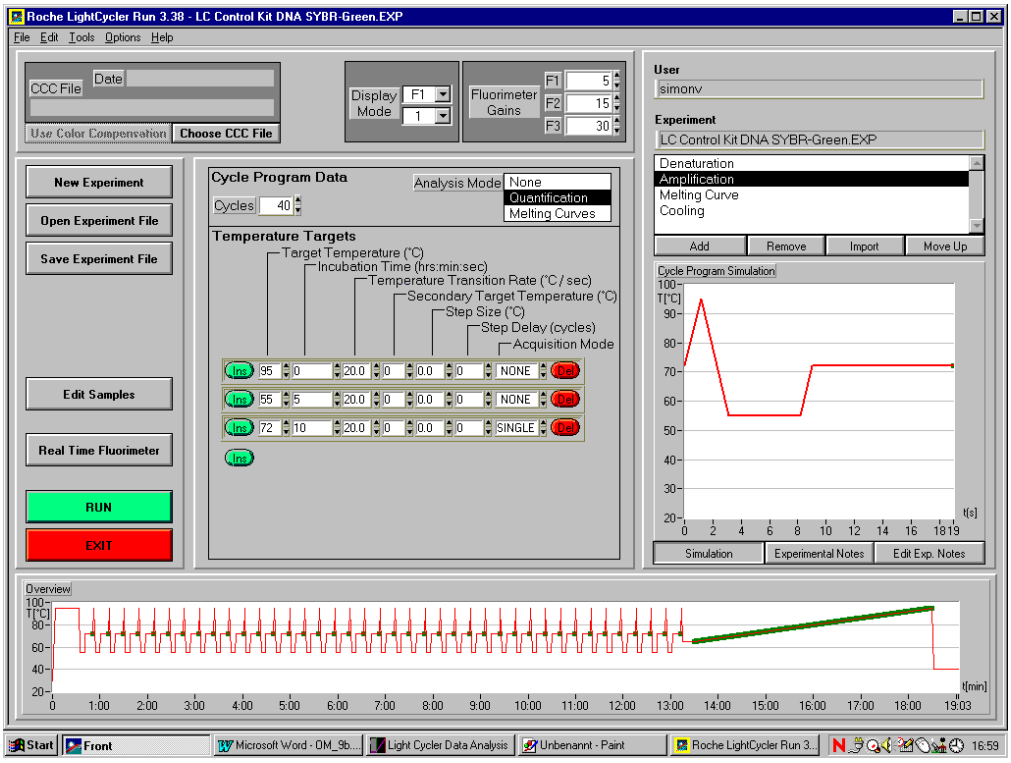
2.1. Programming Screen, Continued

Programming Screen



Functions Available on the Programming Screen

The rest of the topics in Section 2.1 of this chapter describe the software functions that may be activated by either using the menu bars or clicking on various buttons available on the Programming Screen**. Menu options are listed in the top-to-bottom order in which they appear on each menu. Function buttons are described according to the screen areas in which they appear.**

Note: **Use the** Programming Screen **diagram above to locate the screen areas, menus, and buttons as they are described below.**

Defining Cycle Programs

Use the Programming Screen **to define the cycle programs for an Experimental Protocol (*.**exp**-file). Once you have defined your Experimental Protocol, you will be ready to run a LightCycler experiment.**

Tip: **You can use the descriptions in the following sections to familiarize yourself with the actions necessary to define an Experimental Protocol. For instance, as you read the descriptions below, you may activate each function by clicking on the menu option or button, and then enter typical experimental parameters in the tables or fields that appear.**

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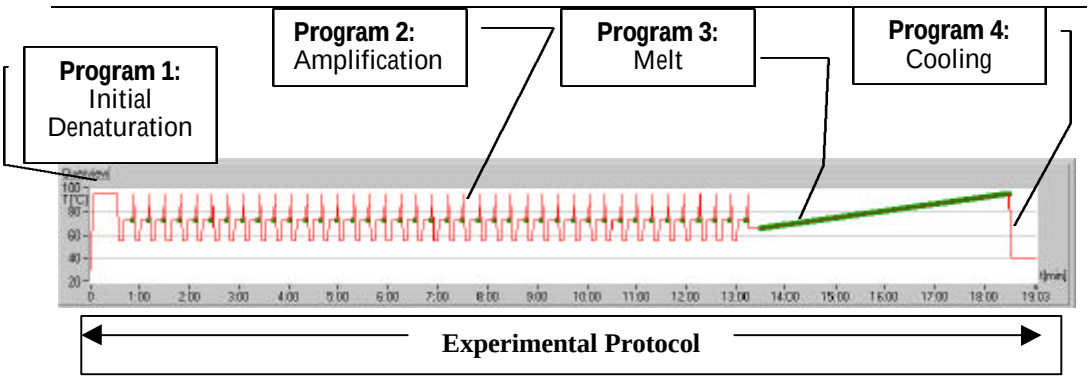
2.1. Programming Screen, Continued

Definitions: Protocol / Program / Segment



An Experimental Protocol **in general contains one or more** Cycle Programs. **In the PCR profile shown above, a typical** Experimental Protocol **contains four programs: 1) an Initial Denaturation Program, 2) an Amplification Program, 3) a Melting Program and 4) a Cooling Program.**

A Program **(e.g. Cycle Program) contains several Temperature** Segments**, each of which defines the time and temperature parameters that will be used for denaturation, annealing, extension and/or melting, cooling as well as the fluorescence acquisition mode used to monitor the amplification signal.**

2.1.1. Menu Options Available on the Programming Screen

Options Available on the Programming Screen Menus

The Menu **bar on the** Programming Screen **offers a variety of options for programming, managing, and displaying Experimental Protocols (*.**exp-files). The following list gives you a brief description of the menu functions.** *Note*: **You may also activate a number of the options listed on these menus key by clicking the buttons found in various areas on the screen. The use of these buttons is discussed in detail in later sections of this chapter.**

Menu	Offers these options
File	Allows you to: • **Create** or **Open** existing *.**exp-**files, • **Save** the current *.**exp-**file, • **Print** the current window contents, and • **Exit** the **Run** program. ***Note*:** Experimental files (*.**lin-**files**)** made with older versions of LightCycler software will be automatically converted to *.**exp-**files when they are first opened. If you had used the Lightcycler Software 1.2x, we recommend converting all *.lin-files into *.exp-files and then deleting the *.lin- and *pro-files. As a *.pro-file may have been used by several *.lin-files, *.pro-files should only be deleted after converting all *.lin-files.
Edit	Allows you to: • **Cut**, **Copy**, and **Paste**, • **Edit Sample Defaults** , • Display **Cycle Program Simulation**, • **Edit Experimental Notes** to your *.**exp-**files, and • **Show Experimental Notes**. ***Note:*** The **Cycle Program Simulation** and **Experimental Notes** will appear in the window in the right portion of the **Programming Screen** under the **User / Experiment** window.

Continued on next page

2.1.1. Menu Options Available on the Programming Screen,
Continued

Options Available on the Programming Screen Menus **(continued)**

Menu	Offers these options
Tools	Allows you to: • Display the **Real Time Fluorimeter**, and • Activate a **Service Utility** function. ***Note:*** 1) The **Real Time Fluorimeter** option permits continuous fluorescence measurement of samples to allow adjustment of fluorimeter gain by the user. 2) The **Service Utility function** facilitates adjusting and testing to permit the instrument to be serviced by an authorized specialist from Roche Molecular Biochemicals Technical Support. ***Caution:*** **Do not activate the Service Utility function unless Roche Molecular Biochemicals service personnel ask you to do so. Settings of the Service Utility function will not be stored and used by the Run program.**
Options	Allows you to: • **Choose (color compensation,-*.ccc-)-files** containing color compensation data, or • **Disable (color compensation,-*.ccc-)-files**, • **Set LED Power** (to turn the LED light source on and off), • **Edit Sample Defaults** (to specify and alter sample information), or • **Use Seek Threshold**.

Continued on next page

2.1.1. Menu Options Available on the Programming Screen,
Continued

Options (continued)

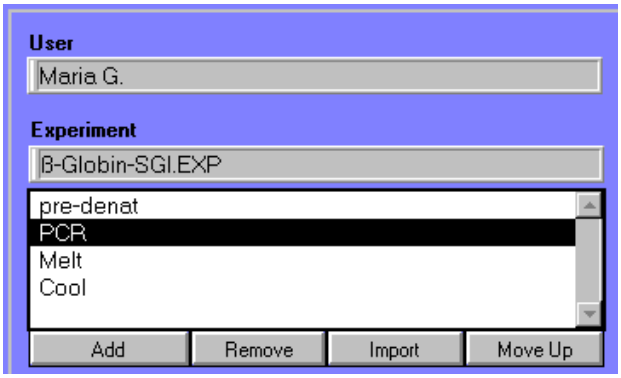
Menu	Offers these options
Options	***Note:*** 1) **Choose (color compensation, -*.ccc-)-files** activates the **Color Compensation** function for use with multicolor reaction systems (see Section 4.3.3 of this chapter and Section 5 of Chapter D for more information). 2) The numerical value below the instrument switch indicates the current LED level on a scale of 0-100%. If the **Calibrated** box is checked, the LED power is set to 75% and cannot be adjusted. If the **Calibrated** box has been cleared, choosing **Set LED Power** causes a yellow box to be displayed on the Programming Screen**,** indicating the LED light source level chosen and allowing the value to be changed**.** ***Note*: We strongly recommend to use Calibrated LED.** 3) The **Use Seek Threshold** function may be inactivated when a capillary, containing no fluorescent dye is not found by the "Seek" function at the beginning of a calibration run.
Help	Allows you to • Access the **Online LightCycler Manual**. *Note*: The Operator Manual can be called by Acrobat Reader. Acrobat reader 4.0 is provided as part of the Software CD.

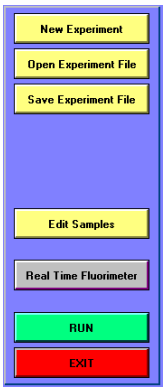
2.1.2. Programming Screen: User / Experiment Window

Location of Window

The User / Experiment Window **is in the right upper corner of the Programming Screen.**

Contents and Functions of User / Experiment Window





- The following information is available in the **User / Experiment Window** (left photo above):
- The **User** name that you used to log onto the LightCycler software appears in the **User** Name Field.
- The **Experiment** Field displays the name of the Experimental Protocol (*.**exp**)-file that is currently loaded into the programming software.
- The **Cycle Program** list (below the Experiment Field) displays the names of the cycle programs contained in the active *.**exp-**file.
- Use the **Cycle Program** list in the **User / Experiment Window**, along with the buttons below the list to:
- Define a new *.**exp-**file, or
- Modify an already existing *.**exp-**file.
  Note: See the next page for a description of the functions activated by these buttons.

Caution: Although you can edit an *.**exp-**file in this window, you must use another window to open or create that file. Go to the left side of the Programming Screen, find the **Experiment / RUN / EXIT** window (right photo above) and:

- Click on the **Open Experiment File** button to open an existing file that you want to edit, or
- Click on the **New Experiment** button to create a new *.**exp-**file.

Continued on next page

2.1.2. Programming Screen: User / Experiment Window, Continued

Cycle Programs

As mentioned in Section 2.1 of this chapter, each Experimental Protocol (*.exp-file) contains one or more cycle programs that are defined by the user, e.g.:

Cycle Program No.	Example of operation described in program
1	Initial denaturation
2	PCR amplification, with repeated cycles of denaturation, annealing, and elongation
3	Melting curve for analysis of the PCR data
4	Cooling

Order of Cycle Programs

In the Cycle Program **window, all cycle programs in the current *.exp-file are displayed in the order they will be executed during a run.**

Cycle Program Buttons

Use the buttons below the **Cycle Program** window to perform the following modifications of the programs in a new or existing *.**exp-**file.

Use this button	To modify an *.exp-file in this way
Add	To create a new program in the *.exp-file
Remove	To delete a program from the *.exp-file
Import	To import cycle programs from other *.exp-files
Move Up	To change the order in which programs will be executed

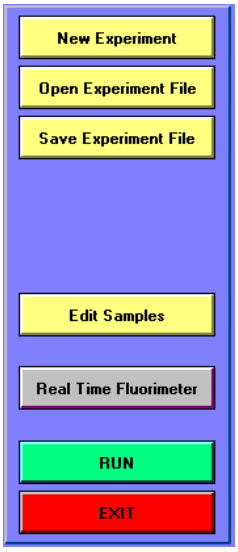
Continued on next page

2.1.3. Programming Screen: Experiment / RUN / EXIT Window

Location of Window

The Experiment / RUN / EXIT Window **is on the left side of the Programming Screen.**

Experiment / RUN / EXIT Window



Functions of Window Buttons

The Experiment / RUN/ EXIT Window **contains seven buttons. Use the buttons to perform the following software functions:**

Use this button	To perform this function
New Experiment	Create a new *.**exp-**file (see Section 2.1.2 above).
Open Experiment File	Select and open an existing *.**exp-**file. ***Note:*** Any ***.lin-**files, used in the previous version of the LightCycler software (Versions 1.2 and 1.22) to contain protocol information, are automatically converted when opened with this button. The *.**lin-**files are copied, converted, and saved with an **exp** suffix.
Save Experiment File	Save a newly defined or modified *.**exp-**file.

Continued on next page

2.1.3. Programming Screen: Experiment / RUN / EXIT Window,
Continued

Functions of Window Buttons (continued)

Use this button	To perform this function
Edit Samples	Name samples in a given run and include additional sample information, e.g. carousel position, type of sample, whether the sample is a replicate of another sample, concentration (if known), and descriptive notes. ***Note:*** See the next section (Section 2.1.4) in this chapter for details on sample information.
Real Time Fluorimeter	Monitor fluorescence over time without running a cycle program. This function allows you to set optimum fluorimeter gain values for your specific protocol. ***Note:*** See Section 2.1.5 in this chapter for details on the Real Time Fluorimeter.
RUN	Start a LightCycler run. ***Note:*** When you click on this button, the Loading Screen window will appear and you will be prompted to enter your sample data.
EXIT	Leave the Programming Screen window.

2.1.4. Programming Screen: Sample Data Entry

Introduction

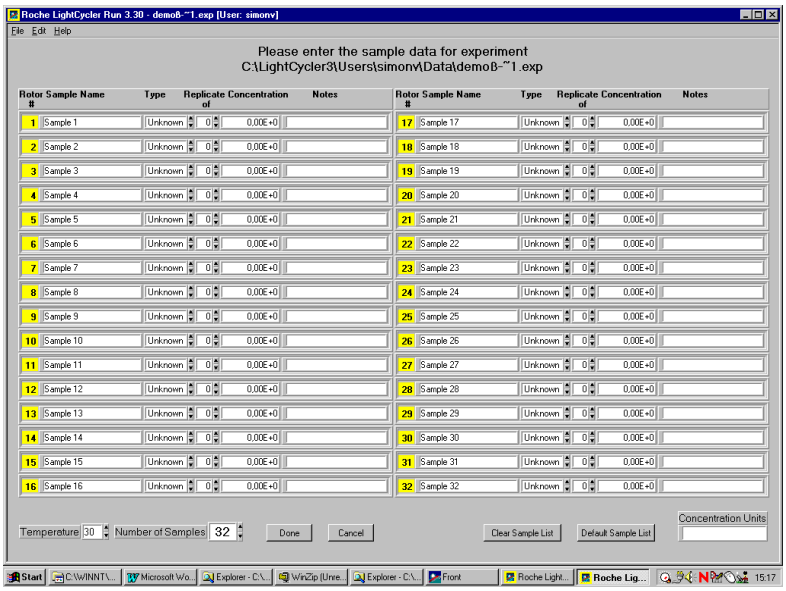
The LightCycler offers you several opportunities to name and describe your samples. You may define sample data in either of two similar windows:

- Click on the **Edit Samples** button in the **Experiment / RUN / EXIT** window (left side of screen).
  Result: The **Loading Screen** will appear. This screen contains a **Sample Data** table (see Section 2.1.4.1 below for details).
  Note: This option allows you to include sample information in the *.**exp-**file so it will be available for every run performed with that file. If you are going to repeatedly run the *.**exp-**file with the same number of samples, use this option.
- Click on the **RUN** button (in the **Experiment / RUN / EXIT** window) to start the LightCycler run. Whether or not you have previously stored sample data in the active *.**exp-**file, the software will prompt you to enter sample data for this particular run.
  Result: The **Loading Screen** with the **Sample Data** table will appear. Note, however, that the options available at this stage differ slightly from those available when the **Edit Samples** button is used (see Section 2.1.4.2 below for details).
  Note: Use this option if the number or the type of samples varies from run to run.

2.1.4.1. Loading Screen Activated by Edit Samples Button

Appearance of Loading Screen Activated by Edit Samples Button

When you click on the Edit Samples Button **in the** Experiment / RUN / EXIT **window, the** Loading Screen **below appears. The software will prompt you to enter your sample default into the** Sample Data **table.**
Note: **Start entering data for carousel position #1 and continue through the last carousel position that contains samples, including any gaps in the sample order.**


Roche LightCycler Run 3.30 - demoß~1.exp [User: simonv]
File Edit Help
Please enter the sample data for experiment
C:\LightCycler3\Users\simonv\Data\demoß~1.exp
Rotor Position # | Sample Name | Type | Replicate of | Concentration | Notes
Temperature 30
Number of Samples 32
Done
Cancel
Clear Sample List
Default Sample List
Concentration Units

Fields in Sample Data Table

To define sample data that will become a part (i.e., the sample default data) of the *.exp**-file, describe a sample set in these fields of the** Sample Data **table**:

This field	Contains this sample data
Rotor Position	**Indicates the position of the sample in the 32-sample carousel.**
Sample Name	**Lists the name of the sample as it will appear in the sample loading dialog and the analysis results.**
Type	**Indicates whether a sample is a positive control, negative control, quantification standard, or an unknown.**
Replicate of	**Indicates whether a sample is a replicate of another sample located at another position in the carousel.** *Note:* **Enter the carousel position of the replicated sample.**
Concentration	**Lists the concentration of a sample if it is known, e.g. if the sample is a quantification standard.**
Notes	**Allows additional sample information to be included.**

Continued on next page

2.1.4.1. Loading Screen Activated by Edit Samples Button,
Continued

Additional Fields on This Loading Screen

After entering the sample description into the fields of the Sample Data **table, enter additional sample information into three fields along the bottom of the** Loading Screen**:**

Field	Location	Contains this information
Temperature	Left lower corner of Loading Screen	Lists the temperature that the sample chamber holds during the "Seek Process" at the beginning of a run. As a default during editing the sample list the LightCycler holds a temperature of 30°C. ***Examples:*** For instance when preparing for an RT-PCR, the temperature during editing the sample list and the subsequent seek may be set to a higher temperature (e.g. 55°C). Higher temperature during the seek may also be set, when using low concentrations of probes, that are fluorescent only at higher temperatures (e.g. beacon probes).
Number of Samples	Left lower corner of Loading Screen	Lists the total number of samples in the carousel. *Note*: If you want to leave space in between the capillaries, indicate the number of samples and the number of free positions in between the capillaries.
Concentration Units	Right lower corner of Loading Screen	Lists the units of concentration to be used during data analysis (i.e., the units appropriate for the values in the **Concentration** fields). *Note*: This information is only used to correctly label the axis of the analysis program. It has no relevance for calculation of data as such.

2.1.4.1. Loading Screen Activated by Edit Samples Button,
Continued

Additional Functions on This Loading Screen

This Loading Screen **contains four buttons that perform the following useful functions:**

Use this button	To do the following
Done	**To confirm the values entered in the sample data fields and return to the Programming Screen.**
Cancel	**To interrupt the entry of sample data.**
Clear Sample List	**To make all fields in the** Sample Data **table blank.**
Default Sample List	**To reset the values in the** Sample Data **table to values previously defined and stored in the *.**exp-**file by the user.**

2.1.4.2. Naming Samples at the Beginning of a Run

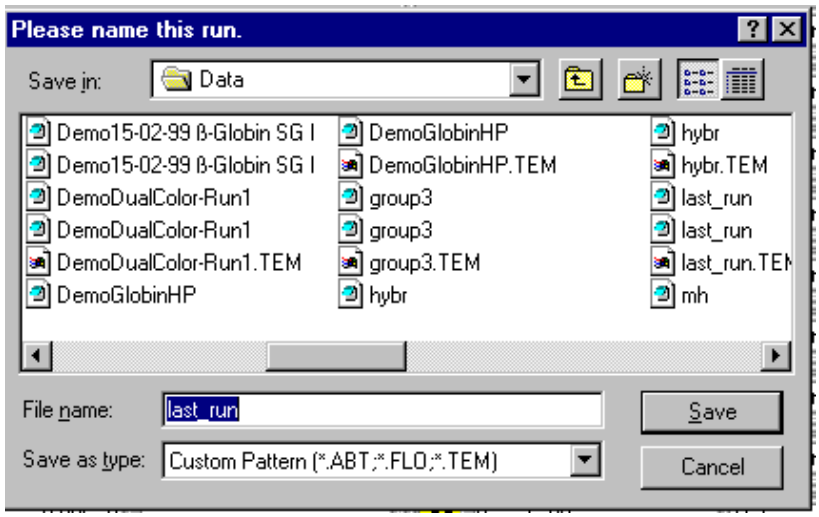
Introduction

When you start a run (by clicking on the RUN **button in the** Experiment / RUN / EXIT **window, the software will offer you an opportunity to enter descriptions of the samples in this particular run.**

Note: **This option is useful if you have not previously defined a default set of samples in the active *.**exp**-file. Even if you have previously defined the samples in the active *.**exp**-file, you may wish to define a different sample set that is unique to this run.**

Create a Run Data File

- After you click on the **Run** button, a dialog box (shown below) will open and prompt you to name the file where the run data will be stored.
- Enter a name for the run data file and click the **Save** button.
  Note: To close the dialog box without specifying a file name, click on the **Cancel** button.



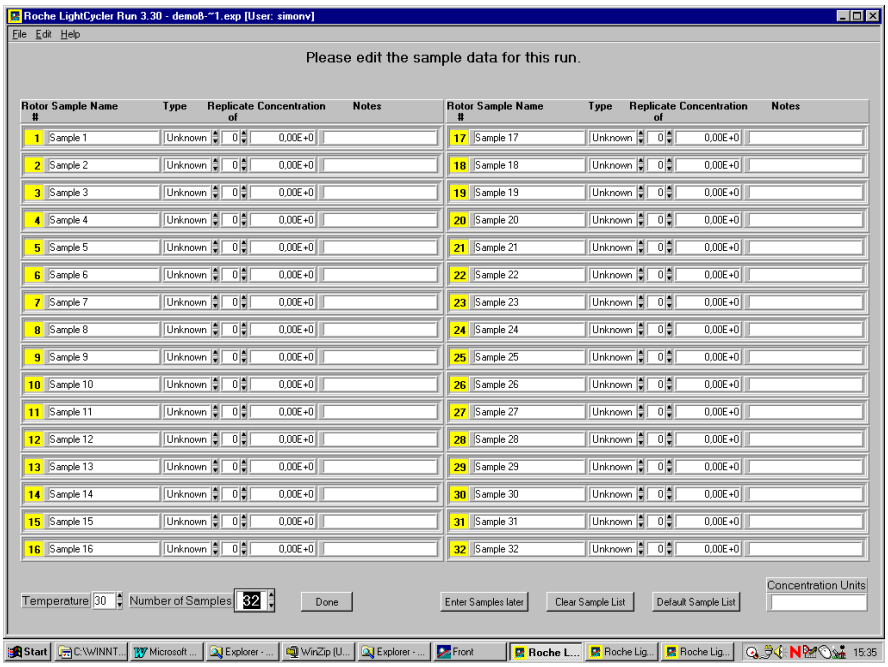
Note: After you save the data run file and the dialog box closes, the **Loading Screen** will appear and the software will prompt you to enter sample data for the current run (see next page).

Continued on next page

2.1.4.2. Naming Samples at the Beginning of a Run, Continued

Loading Screen that Appears at the Beginning of a Run

After you click on the RUN **button in the** Experiment / RUN / EXIT Window**, the** Loading Screen **(shown below) will appear. The software will prompt you to enter sample data for this run in the** Sample Data **table.**
Note: **Start entering data for rotor position #1 and continue through the last rotor position that contains samples, including any gaps in the sample order.**


Roche LightCycler Run 3.30 - demo8-~1.exp [User: simonv]
File Edit Help
Please edit the sample data for this run.
Rotor # Sample Name Type Replicate of Concentration Notes
Temperature 30
Number of Samples 32
Done
Enter Samples later
Clear Sample List
Default Sample List
Concentration Units

Fields in Sample Data Table

To define sample data for the current run, describe your samples in these fields of the Sample Data **table**:

This field	Contains this sample data
Rotor Position	**Indicates the position of the sample in the 32-sample carousel.**
Sample Name	**Lists the name of the sample as it will appear in the sample loading dialog and the analysis results.**
Type	**Indicates whether a sample is a positive control, negative control, quantification standard, or an unknown.**
Replicate of	**Indicates whether a sample is a replicate of another sample located at another position in the carousel.** *Note:* **Enter the carousel position of the replicated sample.**
Concentration	**Lists the concentration of a sample if it is known, e.g. if the sample is a quantification standard.**
Notes	**Allows additional sample information to be included.**

Continued on next page

2.1.4.2. Naming Samples at the Beginning of a Run, Continued

Additional Fields on This Loading Screen

After entering the sample description into the fields of the Sample Data **table, enter additional sample information into three fields along the bottom of the** Loading Screen**:**

Field	Location	Contains this information
Temperature	Left lower corner of Loading Screen	Lists the temperature that the sample chamber holds during the "Seek Process" at the beginning of a run. As a default during editing the sample list the LightCycler holds a temperature of 30°C. ***Examples:*** For instance when preparing for an RT-PCR, the temperature during editing the sample list and the subsequent seek may be set to a higher temperature (e.g. 55°C). Higher temperature during the seek may also be set, when using low concentrations of probes, that are fluorescent only at higher temperatures (e.g. beacon probes).
Number of Samples	Left lower corner of Loading Screen	Lists the total number of samples in the carousel. *Note*: If you want to leave space in between the capillaries, indicate the number of samples and the number of free positions in between the capillaries.
Concentration Units	Right lower corner of Loading Screen	Lists the units of concentration to be used during data analysis (i.e., the units appropriate for the values in the **Concentration** fields). *Note*: This information is only used to correctly label the axis of the analysis program. It has no relevance for calculation of data as such.

2.1.4.2. Naming Samples at the Beginning of a Run, Continued

Additional Functions on This Loading Screen

This Loading Screen **contains four buttons that perform the following useful functions:**

Use this button	To do the following
Done	**Closes the Loading Screen and opens the Run Screen.** *Note:* **The instrument will begin to search for the indicated number of samples.**
Enter Samples Later	**Allows user to define sample data after the beginning of the run.** *Note*: **The** Edit Samples **button on the Run Screen will flash red as a reminder that sample data still must be entered.**
Clear Sample List	**Makes all fields in the** Sample Data **table blank.**
Default Sample List	**To reset the values in the** Sample Data **table to values previously defined and stored in the *.**exp-**file by the user.**

2.1.5. Real Time Fluorimeter (RTF)

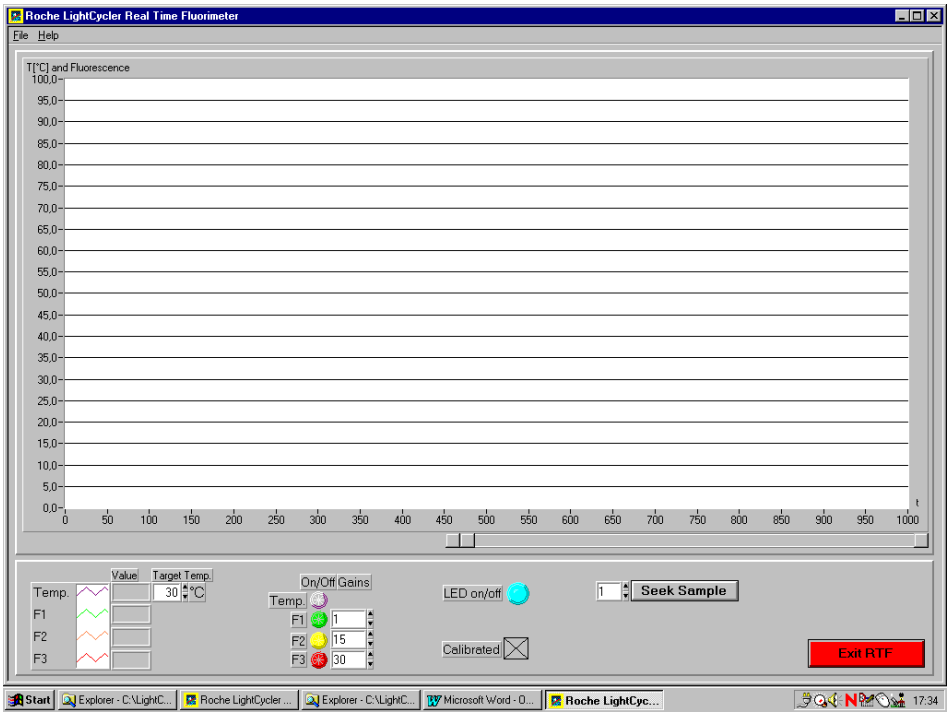
Introduction

The Real Time Fluorimeter (RTF) **permits continuous monitoring of fluorescence, even when a cycle program is not running.**

Whenever you set up a new experiment, e.g. with new primers, nucleic acid templates, or with a new detection format, use the RTF to optimize the measuring sensitivity of the LightCycler fluorimeter. For example, you may adjust the fluorimeter gains to optimize the fluorescence response to variations in temperature or illumination.

Real Time Fluorimeter (RTF) Screen

From the Programming Screen, **click on the** Real Time Fluorimeter **button. The RTF screen will appear (as in the photo below)**



RTF Data Display

The graph that will appear on the gridlines of the RTF screen shows temperature and fluorescence output of a sample over time. The Real Time Fluorimeter reads the raw output from the fluorimeters, so fluorescence outputs are plotted in tenths of a volt (i.e. a reading of 67 units equals 6.7 volts). Time is measured in seconds.
Note: You may adjust the scale on the temperature axis by selecting the upper limit on the graph and typing in a new upper limit value. You may use the scroll bar at the bottom of the screen to move the display back and forth along the time axis.

Continued on next page

2.1.5. Real Time Fluorimeter (RTF), Continued

RTF Settings **Use the fields at the bottom of the RTF screen to enter all RTF settings described below. Fields are listed as they appear on screen from left to right.**

Use this field...	To alter this value
Target Temp.	Changes the temperature in the thermal chamber. The current temperature in the thermal chamber is displayed in the **Value** field to the left of **Target Temp**. ***Note:*** To adjust the temperature, you may either type in a value, or adjust the value in the window incrementally by clicking on the small arrow tabs. ***Caution****:* You must press **Enter** on the keyboard after typing in a temperature.
Gains	Changes the enhancement factors in the fluorescence measuring channels. The range is 1-256. The default setting is 1, 15 and 30 for the respective channels F1, F2 and F3. Each channel can be individually switched on and off by clicking on the colored **On/Off** radio button to the left of the **Gains** field. ***Note:*** As in other data screens, Channel 1 (F1) is optimized to detect emissions from SYBR Green I. Channel 2 (F2) is optimized to detect emissions from LC-Red 640. Channel 3 (F3) is optimized to detect emissions from LC-Red 705. The **colored lines** in the left lower corner of the screen show the color in which each parameter is displayed on the graph.
LED	Turns the LED light source on and off. The numerical value below the switch indicates the current LED level on a scale of 0-100% ***Note:*** . We recommend using Calibrated LED only. If the **Calibrated** box is checked, the LED power is at 75% and cannot be adjusted
Calibrated	Determines the power of the LED. ***Note:*** We recommend leaving the LED power at its normal setting by leaving the **Calibrated** box checked ☒. If you would like to change the power setting, click on the ☒ to clear the box; a scroll box will appear which allows you to make the necessary changes.
Seek Sample	When accessing the RTF, the LightCycler will seek sample #1. Enter the number of sample you wish to be graphed in the data display. Click on the Seek Sample button and the LightCycler will move the indicated sample into the optimal measuring position and acquire any other fluorescence signal of that sample.

Continued on next page

2.1.5. Real Time Fluorimeter (RTF), Continued

Finding the Optimum Gain Setting

To find the optimum gain setting for your experiment, proceed as described below:

Step	Action
1	Prepare a capillary containing a complete PCR reaction mixture. ***Note:*** As template, use the highest starting nucleic acid concentration that you plan to use in your experiment.
2	• Place the capillary into the carousel. • Load the rotor into the LightCycler.
3	Depending on the detection format you plan to use, enter the temperature at which the fluorescence will be acquired during your experiment. ***Example:*** For SYBR Green I format, set the temperature at 72°C (temperature at end of extension phase). For Hybridization Probes format, set the temperature at the annealing temperature of the probes (usually in the range of 50°-60°C, at the end of annealing phase).
4	Verify that the correct measuring channel is turned on (i.e., the correct LED is lit). ***Note:*** Click on the **F1** button for SYBR Green I; **F2** button for Hybridization Probes.
5	Click on the **Seek Sample** button to bring the capillary into the focus of the fluorimeter.
6	**Adjust the fluorimeter** Gain **(by clicking on the small arrow tabs) until the displayed fluorescence signal is between 10 and 20.** *Caution:* **Gain values determined in the RTF will not be saved automatically when you exit the RTF and return to the** Programming **or** Front Screen**. However when returning to the Programming Screen you will be asked to confirm whether you wish to use the settings, chosen in the RTF in your experiment.**

Exit RTF

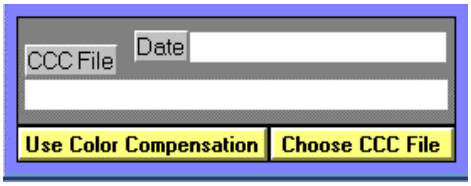
To quit the RTF and return to the Programming Screen **or** Front Screen**, either click on the** Exit RTF **button or choose "Exit RTF" from the** File **menu.**

2.1.6. Programming Screen: Color Compensation Window

Location of Window

The **Color Compensation Window** is in the upper left corner of the Programming Screen.

Color Compensation Window



Function of Color Compensation Window

The **Color Compensation Window** allows you to select a software file (*.**ccc** - file) containing information which can improve the display of data obtained with multicolor analysis systems (e.g., Dual Color Detection experiments). The use of color compensation is restricted to Hybridization probes. The DNA binding dye SYBR Green I is analyzed in channel 1 with no color compensation.
Color compensation is also not required when a single color detection with LC Red 640 or LC Red 705 is performed.
Note: See Section 5 of Chapter D for an example of a system that uses color compensation files.

How to Load a ccc File

If you select a *.**ccc-**file in the **Color Compensation Window**, color compensated run data will be displayed during a PCR run. To view the effect of color compensation during a run, do the following:

Step	Action
1	In the **Programming Screen**, open the *.**exp-**file (i.e., file name.exp).
2	In the **Color Compensation Window,** click on "**Choose CCC File"** and select the appropriate color compensation file from the Calibration directory window. Color Compensation is automatically enabled.
3	Click on **Use Color Compensation** to disable Color Compensation.

Note:

- You may also choose and/or disable and enable Color compensation during a Run.
- When Color Compensation is chosen, the Color Compensation file content will be stored as part of the data-file, generated during the a Run.
- The Display of Fluorescence History however will not switch the displayed fluorescence values back to the uncorrected values if you turn color compensation off. Data will be stored without color compensation after the run.

2.1.6. Programming Screen: Color Compensation Window,
Continued

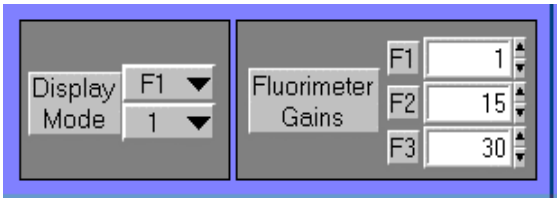
Use of *.ccc - Files for Data Analysis	In addition to using a *.**ccc-**file to generate a color compensated run display, you can use the file to analyze multicolor experiments more accurately. This important use of *.**ccc-**files is described more fully in Section 4.3.3 of this chapter.

2.1.7. Programming Screen: Display Mode / Fluorimeter Gains Window

Location of Window

The **Display Mode / Fluorimeter Gains Window** is in the upper center portion of the Programming Screen.

Display Mode / Fluorimeter Gains Window



Display Mode

To select the fluorescence display used during LightCycler runs, use the pull-down menus in the **Display Mode** window. One menu sets the numerator of the selection; the second sets the denominator of the selection. Choices for numerator and denominator include F1, F2, F3, and 1.

Note: The F1 channel is optimized to detect emissions from SYBR Green I or fluorescein, at wavelengths around 530 nm. The F2 channel detects emissions from LC-Red 640. The F3 channel is optimized to detect emissions from LC-Red 705 (see Section 1.3.2 of Chapter C).

Examples: Use the following settings for typical experiments:

- For quantification runs with SYBR Green I alone, select F1/1.
- If fluorescein is used alone, select F1/1.
- If fluorescein is used with another fluorophore as a FRET pair (see Section 1.3.2 of Chapter C), select either F2/1 or F3/F1, depending on whether the second fluorophore is LC-Red 640 (F2/1) or LC-Red 705 (F3/F1).

Note: Channel Settings may also be selected and altered during a Run.
For more information on the use of these fluorescence display settings in various applications, see Chapter D.

Continued on next page

2.1.7. Programming Screen: Display Mode / Fluorimeter Gains Window, Continued

Fluorimeter Gains

Different applications require different detection sensitivity. Use the **Fluorimeter Gains** window to set the correct detection sensitivity for your application in each of the fluorimeter channels. The gains may be adjusted on a scale from 1 to 256. You may either type in a gain number or click on the small tabs to adjust the number gradually. The default setting is 1, 15 and 30 for the respective channels F1, F2 and F3.

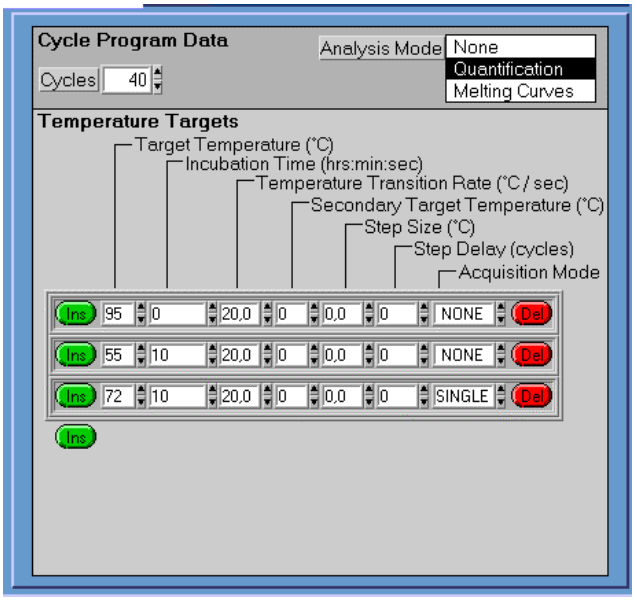
Note: Grain Settings cannot be changed during a Run, but must be set to their optimum values as described earlier. To determine the correct gain experimentally, use the **Real Time Fluorimeter (RTF)** (see Section 2.1.5 of this Chapter). If the signal for a channel in the RTF is too high, select a lower gain number. If the signal level is too low, select a higher gain number.

2.1.8. Programming Screen: Cycle Program Data and Temperature Targets Window

Location of Window

The **Cycle Program Data and Temperature Targets Window** is in the center portion of the Programming Screen.

Cycle Program Data and Temperature Targets Window



Cycle Program Data

The **Cycle Program Data** section automatically appears when a new cycle program is added to a protocol (e.g. by clicking the **Add** button under the **Cycle Programs** list as described in Section 2.1.2 above).The section accepts the following information:

Part	Option
Cycles Field	Type in the number of cycles to be run with the cycle program parameters to be defined in the **Temperature Targets Window**.
Analysis Mode Menu	Depending on the application and the cycle program, use the pull down menu to select the correct analysis mode: • **None,** • **Quantification,** or • **Melting Curves.** ***Note:*** The default setting is **None**. Change the setting if the data acquired during the cycle program is to be used for quantification or melting curve analysis.

Note: Information entered in this window is automatically saved without onscreen confirmation.

Continued on next page

2.1.8. Programming Screen: Cycle Program Data and Temperature Targets Window, Continued

Temperature Targets

Use the **Temperature Targets** section to define the temperature profiles for each individual program. Type the appropriate settings in the horizontal fields between the **Ins** and the **Del** buttons.
Each temperature segment is inserted or deleted as follows:

Button	Click on the button...
Ins (Green)	To enter a new temperature segment or to add a temperature segment in a new position.
Del (Red)	To delete a temperature segment.

Field Description

Enter the following information into the fields in the **Temperature Targets** section:

Field	Purpose
Target Temperature (°C)	Defines the temperature of the segment in °C, e.g., 94°C for denaturation or 72°C for elongation phase.
Incubation Time (h:min:s)	Defines the holding time of a temperature segment. Use the following guidelines for setting typical incubation times: • For denaturation: 0 s • For annealing with SYBR Green I: 0–10 s • For annealing with hybridization probes: 0–15 s • For elongation: As a general rule, divide the length of fragment in bp by 25 to determine the incubation time (in seconds). ***Note:*** If you enter a single number, it indicates the time is in seconds. To enter a time in minutes, you have to use a colon to separate minutes and seconds, e.g. ”10:30” means 10 minutes and 30 seconds. When entering 60 seconds or more "seconds" when leaving this field an automatic conversion from seconds to minutes occurs (e.g. 100 seconds will be converted to "1:40").
Temperature Transition Rate (°C/s)	Defines rates at which the instrument changes temperature between temperature targets. The slowest rate is 0.1°C/s and the fastest rate is 20°C/s. ***Note:*** A fast transition rate (20°C/s) works well for amplification programs. Slower rates (0.1–0.2 °C/s) are recommended for melting curves.

Continued on next page

2.1.8. Programming Screen: Cycle Program Data and Temperature Targets Window, Continued

Field Description **(continued)**

<table>
<tr><th>Field</th><th>Purpose</th></tr>
<tr><td>Secondary Target Temperature
(°C)</td><td>Defines a second target temperature within a segment beginning at a defined cycle number (see Step Delay below).
<u>Note:</u> This is suitable for a so-called ”touch down” PCR. Here, annealing occurs at the upper temperature limit to ensure specificity during the first 15-20 cycles. However, this reduces the efficiency of amplification. After 15-20 cycles, the annealing temperature is decreased by as much as 10°C to the Secondary Target Temperature where annealing occurs with lower specificity to facilitate greater amplification efficiency.</td></tr>
<tr><td>Step Size
(°C)</td><td>Defines the degree change per cycle used to step up/step down from the Target Temperature to the Secondary Target Temperature.
<u>Note:</u> A decrease of 1°C/cycle is commonly used in ”touch down” PCR.</td></tr>
<tr><td>Step Delay
(cycles)</td><td>Defines the cycle number at which the step up/step down from the Target Temperature to the Secondary Target Temperature begins.</td></tr>
<tr><td>Acquisition Mode</td><td>Defines the time in each PCR cycle at which fluorescence measurement of samples is made. The available choices are:
<table>
<tr><td>None</td><td>No fluorescence measurement.</td></tr>
<tr><td>Single</td><td>Measures fluorescence once at the end of the temperature segment selected. For the Hybridization Probe format, measurement occurs at the end of annealing phase; for the SYBR Green I format, it occurs at the end of the elongation phase.</td></tr>
<tr><td>Cont.
(continuous)</td><td>Monitors fluorescence data continuously from the first sample to the last one. The temperature continues to increase during measurement.
<u>Note:</u> This acquisition mode is suitable for melting curve analysis.</td></tr>
</table>
</td></tr>
</table>

Continued on next page

2.1.8. Programming Screen: Cycle Program Data and Temperature Targets Window, Continued

Field Description **(continued)**

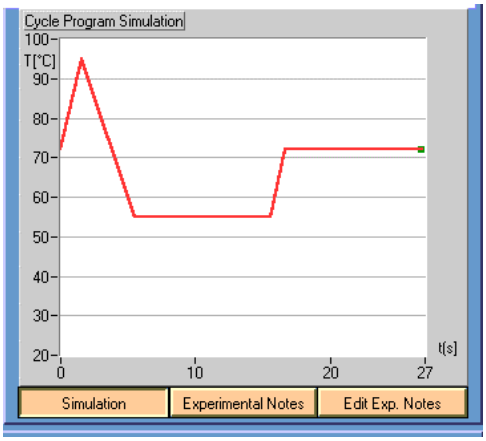
Field	Purpose		
Acquisition mode (continued)	**Step** (stepwise)	Measures fluorescence at each temperature transition. ***Example:*** If you use **Step** to acquire fluorescence during a melting curve analysis, enter 0.2°C/s as your **Temperature Transition Rate. T**he system will heat up the samples by 0.2°C, hold the temperature and measure all samples. It will then stepwise increase the temperature by 0.2°C and measure at the respective temperatures. ***Note:*** This acquisition mode is suitable for melting curve analysis.	

2.1.9. Programming Screen: Graphic Simulation Window

Location of Window

The **Graphic Simulation** window is just below the **Cycle Program** list on the right side of the **Programming Screen.**

Graphic Simulation Window



Cycle Program Simulation

Click on the **Simulation** button in the **Graphic Simulation** window to display the **Cycle Program Simulation**. This simulation shows a graph of a single cycle of the program named in the **Cycle Program** list (directly above the window). Use this visual aid to confirm your cycling parameters

Note: In the simulation, the temperature profile is displayed as a red line, while the fluorescence acquisition profile is graphed in green.

Experimental Notes

In addition to the **Cycle Program Simulation** display, the **Graphic Simulation Window** can display notes about the current program. Use the buttons beneath the window to do the following:

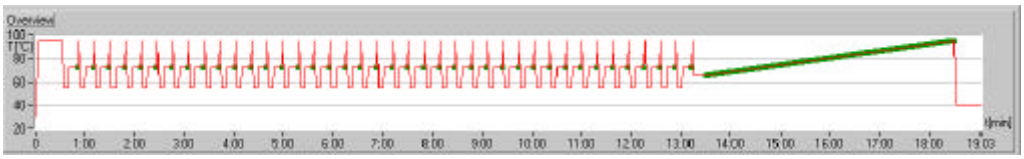
Use this button	To do this
Experimental Notes	Display notes on the program highlighted in the **Cycle Program** list.
Edit Exp. Notes	Alter the notes displayed in the window.

2.1.10. Programming Screen: Cycle Program Overview Window

Location of Window

The **Cycle Program Overview** window stretches across the bottom of the **Programming Screen.**

Cycle Program Overview Window



Temperature Profile vs. Time

The **Cycle Program Overview** window displays a graph of the current experimental protocol. The temperature profile is graphed in red, while the fluorescence acquisition profile is graphed in green. The time for the programmed Experimental Protocol is indicated as rough estimate.

3. Running the LightCycler

3.1. LightCycler Run Screen

Introduction

When a PCR run is started, the monitor switches from the **Programming Screen** to the **Run Screen**. This screen displays actual fluorescence values, the development of fluorescence during the run, and the actual temperature online.
Note: A picture of the **Run Screen** is shown on the next page.

Run Screen Display

After the instrument detects the samples, temperature cycling begins and the Run Screen will display three graphs:

- Fluorescence vs. Time (**Fluorescence History**)
- Temperature vs. Time (**Temperature History**)
- Relative fluorescence of each sample (**Current Fluorescence** bar graph).

The screen also displays the following:

- Cycle Program Name
- Cycle Program Segment
- Cycle Number (currently in progress)
- Current Fluorescence Display Mode.
- Samples Names (and the corresponding line color used to display the progress of that sample).

Note: If the LightCycler is unable to detect one or more samples, a dialog box will tell the user how many samples were found at which position and offer a chance to abort the run or continue it.
This behavior also applies, when intentionally one position of the sample carousel was left free.
In case the system was not able to find a capillary, repeat the seek with "Use Seek Threshold" disabled in the Options pull-down of the Programming Screen.
Position #1 is always measured. Here, if no capillary is found, a default position, determined during instrument calibration is used.

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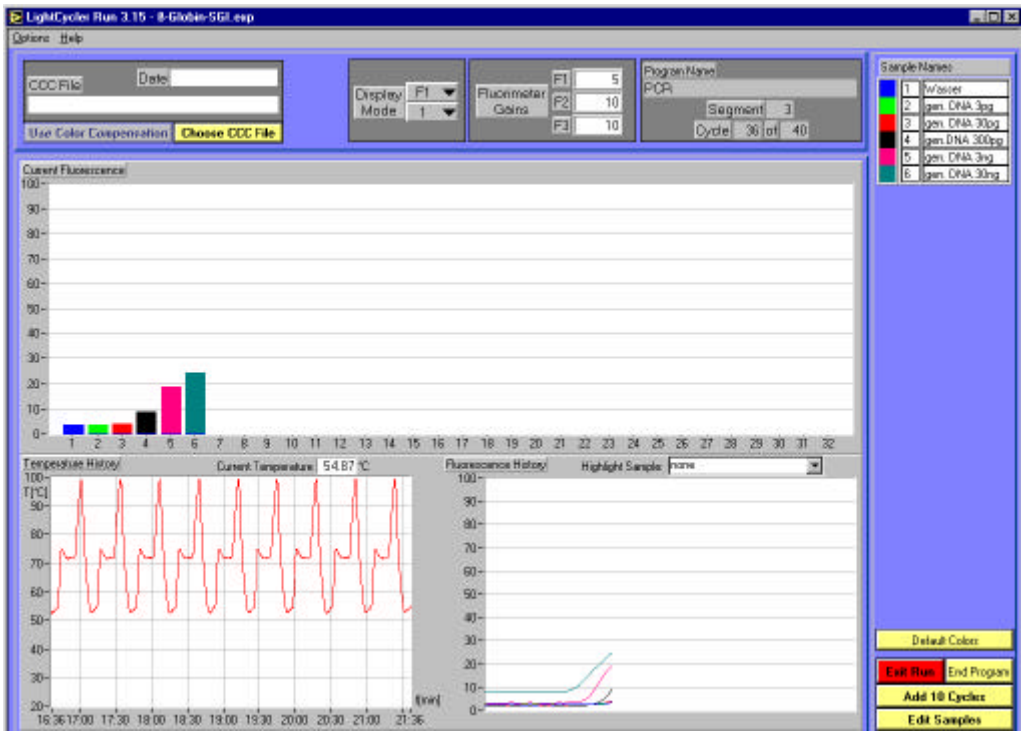
3.1. LightCycler Run Screen, Continued

LightCycler Run Screen



Fluorimetry Controls

A panel at the top of the Run Screen controls several fluorimetry display and acquisition (measurement) parameters.

Area	Function
CCC File (fields)	If you are using a color compensation file, this area will list the name of that file.
Display Mode (pull down menus)	May be used to select the fluorescence channel(s) displayed during LightCycler runs. ***Note:*** Display mode is usually specified during programming (see Section 2.1.7 above), but may be changed here.
Fluorimeter Gains (fields)	Displays the detection sensitivity in each of the fluorimeter channels. ***Note:*** Gains cannot be changed during a run. Gains are set during the programming phase of the experiment (see Section 2.1.7 above).

Continued on next page

Program Name / Segment / Cycle Number

The right section of the top panel shows 1) the name of the program (e.g. PCR, Annealing), 2) the number of the temperature segment now in progress, and 3) the number of the cycle now in progress.

Sample Names/ Default Colors

A window in the far right corner of the screen lists 1) the names of the samples in the current run, 2) the rotor position of each sample, and 3) the color of the line (or bar) that represents each sample in the graphs.

Note: You can change the color coding by just clicking on the color squares to the left of the samples. Changes are effective in the current run and shall not be forwarded to the Graphics Defaults settings.To reset the color defaults that you defined earlier (see Section 1.5 above), click on the **Default Colors** button (lower right corner of the screen). This does not affect data display in the LCDA module.

Other Buttons

In the lower right corner of the screen, there is a set of buttons. Use these buttons to perform the following functions:

Click on this button...	To do this...
End Program	Stop the cycle program that is currently running and move onto the next cycle program. ***Note:*** If the current program is the last in the protocol, clicking this button will return you to the **Front Screen**.
Add 10 Cycles	Add ten more cycles to the program in progress. ***Note:*** Use this button if your amplification reaction is not complete or your reaction is proceeding slowly. This change is only effective for the current run and shall not be stored in the *.exp-file.
Exit Run	End the run immediately and offer you two options: • Save, or • Cancel. ***Note:*** If there was an error in the program or you do not want to save the data click on **Cancel**. Otherwise, click on **Save** to save the run data for later analysis. ***Caution:*** If a protocol is run twice, both sets of data cannot be saved. You can replace the first set of data with the second set, rename the second data set, or hit **Cancel** to save the original data.

Continued on next page

End of Run

- If the LightCycler completes a run, it will automatically save the program.
- After the LightCycler finishes a run, it will automatically open a data analysis window that displays the data acquired in the current run.

Note: When at the end of a Run another experiment is being analyzed, the current data will not be displayed atomatically.

4. Data Analysis

4.1. Introduction

Starting Data Analysis

To begin data analysis, do either of the following:

- Simply allow the LightCycler to complete a run. When the protocol has finished, the software will automatically display the collected data on the **LightCycler Data Analysis (LCDA) Front Screen**.
- If no run has been performed, open the **LCDA Front Screen** by clicking on the **Data Analysis** button on the **LightCycler Front Screen**.

Analysis Formats

The LightCycler Data Analysis (LCDA) software allows a LightCycler user to analyze quantification data or melting curve data acquired during a LightCycler reaction. The LCDA software can display and analyze data in two formats:

Format	Select by clicking	Description
Quantification	**Quantification** button (at top of screen)	Estimates the original number of target DNA copies in a sample by comparing it to at least two known concentrations of a standard. It displays the amplification profile of a PCR.
Melting Curve	**Melting Curve** button (at top of screen)	Displays a fluorescence curve profile obtained during a slow denaturation of PCR products, and includes options for differentiating melting curves to give melting peaks, integrating the area under the melting peaks, and defining the melting temperature of amplified products.

Note: You must select the segment of the run to be analyzed and define other analysis parameters before the data can be analyzed. For more information, see the following sections of this chapter for details:

- See Section 5 for details on defining parameters for the Quantification format, including setting the baseline and crossing line, determining crossing points, and selecting data from a set of standards to generate a standard curve.
- See Section 6 for details on defining parameters for the Melting Points format, which are similar to those used for the Quantification format.

In this Section

The rest of Section 4 gives a brief overview of the various parts of the LCDA Front Screen. See Sections 5 and 6 for detailed information on using these parts to set up a Quantification or Melting Curve analysis.

4.2. Select Data File / Data Analysis Window

Select Data File to Analyze

You must choose the data to be analyzed by doing the following:

- Select "Open" from the **File** menu on the **LCDA Front Screen**. A **Data Analysis Window** (shown below) will open and display a list of LightCycler data files. For each file, the list gives the **File Name, User Name,** date / time **Created,** and date / time **Analyzed**
  Note: You will see only those files that belong to the current user (e.g., the user whose log on name was used to enter the LightCycler software. Every user has an individual data folder.
- Use the pointer to highlight the file containing the data.
- Click on the **Open** button.

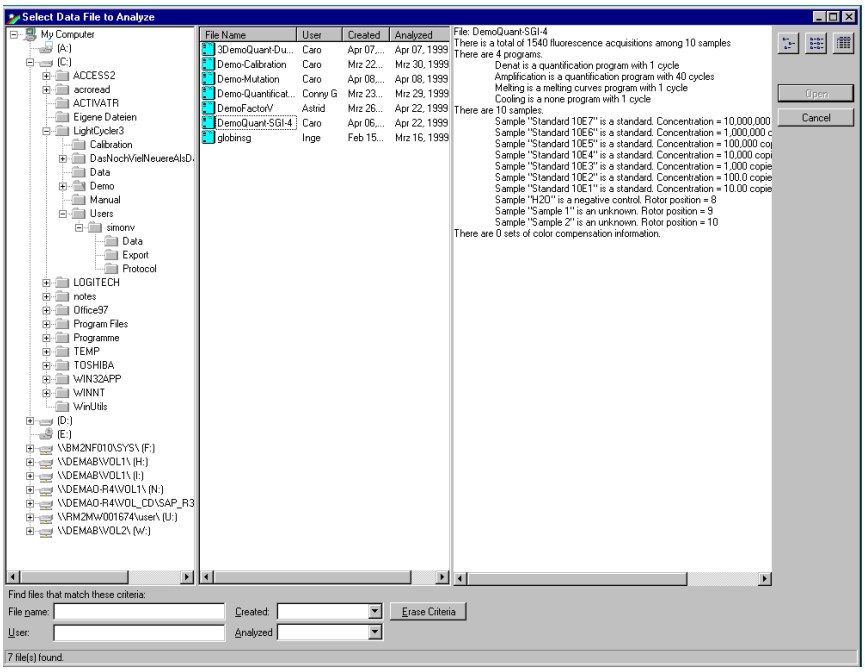
Result: The **LCDA Front Screen** will open and display the selected data file in a new window.

Note: A new window is created for each open file. Thus, you may open and analyze several data sets by moving between windows.

Searching for a Data File

If you are not certain which data file you want, you may use the fields at the bottom of the **Data Analysis Window** to search for a select subset of files. These fields allow you to search for files by **File name**, User name, D**ate Created** or D**ate Analyzed**.

Data Analysis Window



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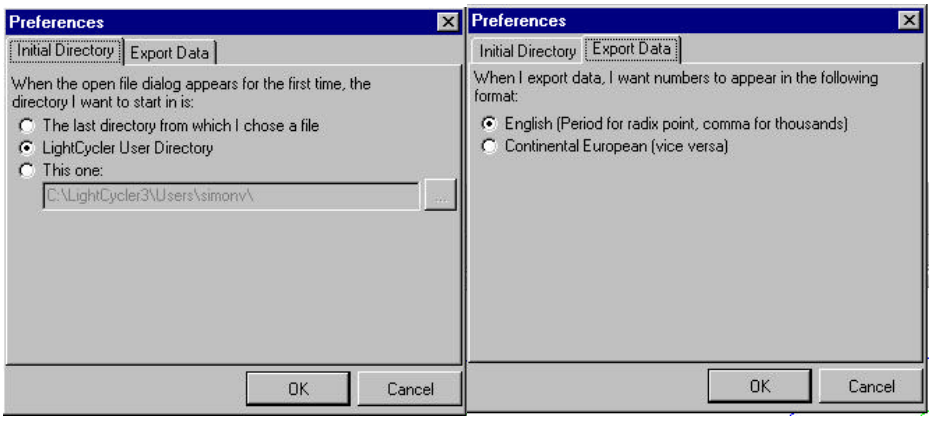
4.2. Select Data File / Data Analysis Window, Continued

Additional Information on Data Files

After a file is highlighted (but before it is opened), additional information about the file will be displayed in the box at the right of the **Data Analysis Window**. This information includes:

- File name
- Number of linked programs that constitute the run, with a brief description of each program
- Number of samples
- List of all sample names.
  Note: If the samples were designated as standards of known concentration, the concentrations will also be listed next to the sample name.

File Open Default Settings


Preferences
Initial Directory
Export Data
When the open file dialog appears for the first time, the directory I want to start in is:
The last directory from which I chose a file
LightCycler User Directory
This one:
C:\LightCycler3\Users\simonv\
OK
Cancel
Preferences
Initial Directory
Export Data
When I export data, I want numbers to appear in the following format:
English (Period for radix point, comma for thousands)
Continental European (vice versa)
OK
Cancel

The Preferences option under the pull down "Help" menu of the LCDA Front screen allows customizing the file open default settings.

Select by Clicking	Description
Initial Directory	Allows the user to define the directory from which he wants to start in when the open file dialog appears for the first time.
Export Data	Allows the user to define the format of numbers when data will be exported.

4.3. LCDA Front Screen

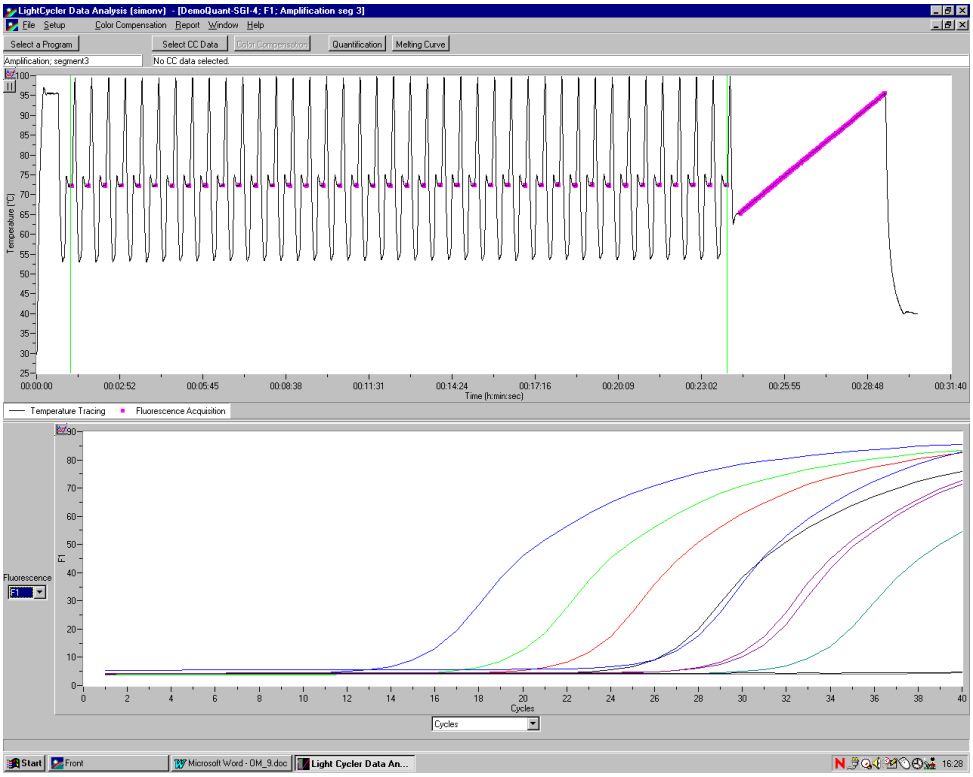
Introduction

When you open a data file from the **Data Analysis Window**, the data will automatically be displayed in the **LCDA Front Screen** shown below. The data will eventually be displayed in two sections of the window:

- Initially, the top section of the window will display the run data as a **Temperature versus Time** graph. On this graph, you must define the section of the run that will be analyzed by bracketing the selected section with the green cursors (see Section 4.3.1).
- After you set the cursors, the lower section of the window will display the fluorescence data acquired during the selected section of the run (as shown below).

Note: In this and all subsequent data analysis screens, you can enlarge or reduce the size of each graph by clicking on the horizontal divider between the graphs and moving it up or down.

LCDA Front Screen



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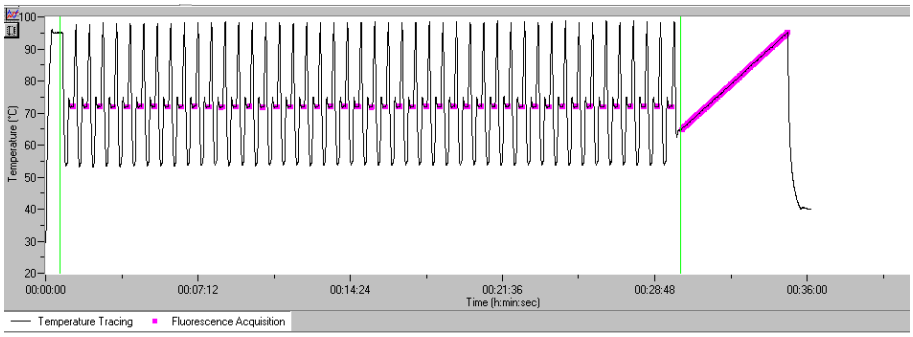
4.3.1. LCDA Front Screen: Temperature vs. Time Graph

Set Cursors for Quantification Analysis

To use Quantification analysis to analyze the data (i.e., data acquired once per cycle), set the green cursors to bracket the section of the graph that represents the amplification program (as shown in the photo below).

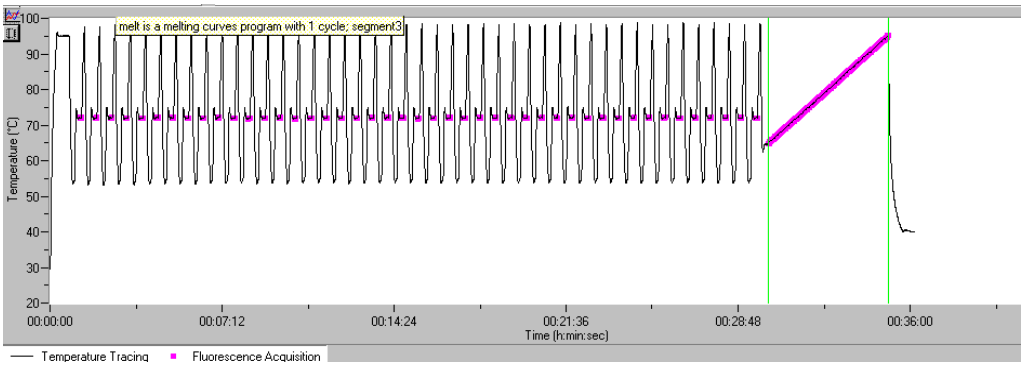
Note: To automatically bracket an amplification program, click the **Select a Program** button and then select the program segment in which amplification took place. (For example, see the upper left corner in the picture of the LCDA Front Screen on the previous page. The name of the selected segment appears in the window below the **Select a Program** button.)
To further refine the data to be analyzed grab and move the green cursors manually.



Set Cursors for Melting Curve Analysis

To analyze melting curve data (i.e., data acquired continuously), set the green cursors to bracket the section of the graph that represents the melt program (as shown in the photo below).

Note: To automatically bracket a melt program, click the **Select a Program** button and then select the program segment of the protocol in which melting took place. To further refine the data to be analyzed grab and move the green cursors manually.



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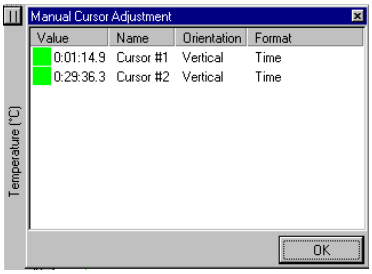
4.3.1. LCDA Front Screen: Temperature vs. Time Graph, Continued

Upper Graph: Temperature vs. Time

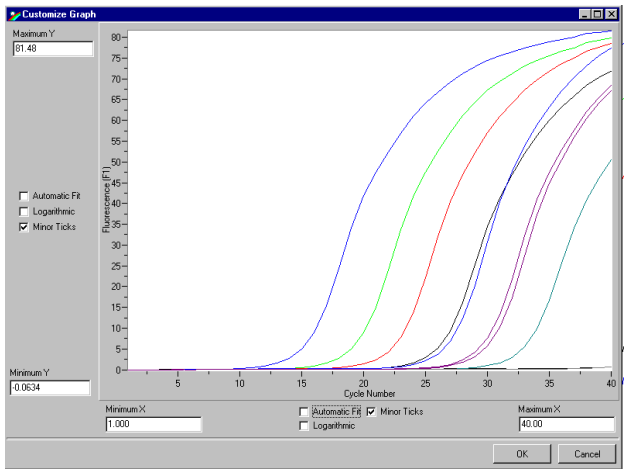
The graph in the top section of the **LCDA Front Screen** displays the temperature cycles (black line) during the run, as well as the temperatures at which fluorescent data was acquired (pink line).
To define the area of this graph that will be analyzed, do one of the following:

- Use the mouse to move the green cursors until they bracket the area to be analyzed.
- Click on the button at the left corner of the graph to open a window (as shown below). In this window, you may enter a number in the **Value** field to specify the position of each cursor (expressed as a particular time, in h:min:s format). After you have entered the times for each cursor, click the **OK** button to set the cursors.



Change Graph Settings

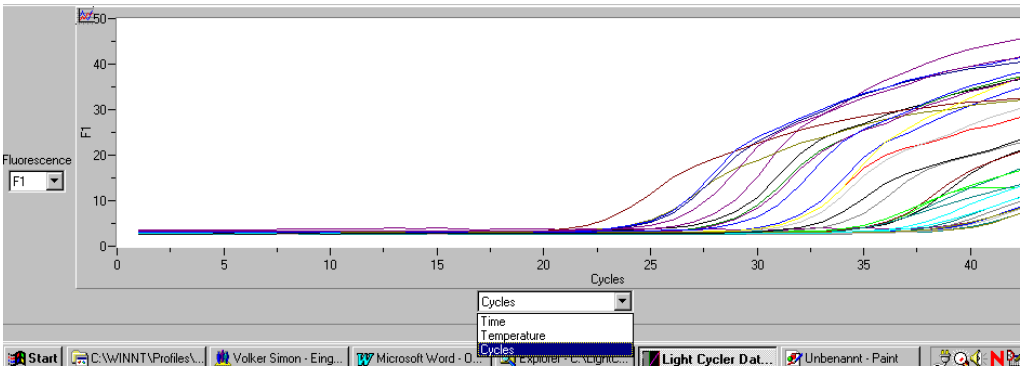
To change any axis values of the graphs displayed click on the button on the top left corner of the graph.
This will bring up a **Customize Graph** screen. In this screen the user can define values for the axis which will then be carried into the LCDA display. Deselect the Automatic Fit option and enter values for the axis in the appropriate fields.



4.3.2. Fluorescence vs. Cycle Number / Time / Temperature Graph

Fluorescence vs. Cycle Number

After you set the cursors in the top graph (see Section 4.3.1 above), the bottom graph (as shown in the photo below) will display the fluorescence data acquired during the bracketed section of the protocol.



Select x-Axis

In the pull down menu below the bottom graph, you may select any of three options for the parameter plotted on the x-axis: **Time**, **Temperature**, or **Cycles**. Use the following guidelines to select the appropriate parameter:

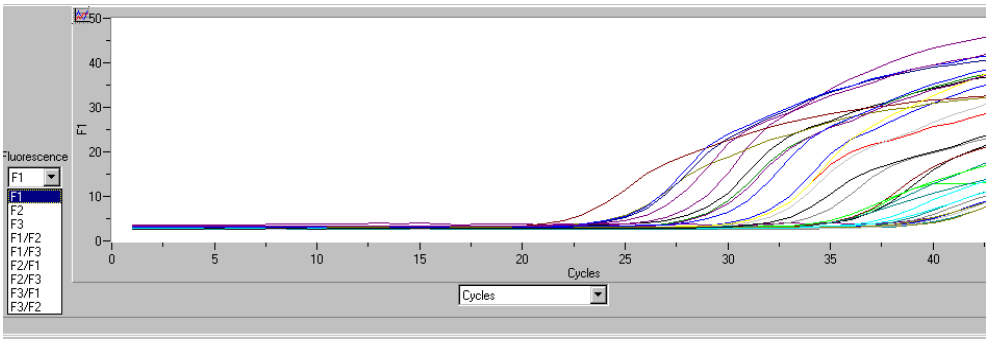
If you will analyze...	Then select...
Fluorescence data acquired once per cycle (i.e., quantification data)	**Cycles**
Fluorescence data from a melt	**Temperature**
Any experiment (default setting, for data viewing only)	**Time**

Continued on next page

4.3.2. Fluorescence vs. Cycle Number / Time / Temperature Graph, Continued

Select Fluorescence Display

To set the fluorescence channel to be displayed in the lower graph, highlight the appropriate item in the pull-down menu to the right of the y-axis. You may choose to display fluorescence data from a single channel (**F1, F2, or F3**) or a ratio of data from two channels (**F1/F2, F1/F3, F2/F1, F2/F3, F3/F1,** or **F3/F2)**.



Guidelines for Fluorescence Display

Use the following guidelines to determine which fluorescence display to use for various applications:

Use this fluorescence display...	For assays containing...
F1	• SYBR Green 1 • Fluorescein
F2 F2/F1	• LC-Red 640 • LC-Red 640 / Fluorescein Hybridization probe pair
F3, F3/F1	• LC-Red 705 • LC-Red 705 / Fluorescein Hybridization probe pair

Note: When opening a file the first program with data acquisition is bracketed and the channel setting is chosen in the *.exp-file selected.

Example: For examples of experiments which use different fluorescence display settings, see the applications described in Chapter D.

4.3.3. LCDA Front Screen: Using Color Compensation Files

Color Compensation

The following dyes can be used for multicolor analysis with the LightCycler**:**

Analysis Format	Channel 1	Channel 2	Channel 3
Hybridization Probes	Fluorescein	LC Red 640	LC Red 705

Each of the fluorophores listed above has a different emission maximum, and the LightCycler detection filters have been optimized to these maxima. However, when more than one of these dyes is used in a single run, there is some crosstalk between fluorimeter channels. LCDA color compensation software compensates for this between-channel crosstalk.

Preparing a Color Compensation File

To use color compensation, you must first create a *.ccc (**ccc)-**file in a calibration experiment with the dyes listed above (see Section 5 of Chapter D for details).
Note: We offer a LightCycler Color Compensation Set (Cat No. 2 158 580), with ready-to-use solutions and optimized conditions for the generation of **ccc** files.

Saving a Color Compensation File

After completing a calibration run, do the following:

Step	Action
1	Open the acquired data in the Programming **Screen**.
2	In the **Select a Program** pull-down menu in LCDA, highlight the program segment that includes the melting program.
3	From the **Color Compensation** menu, select “Calibration”.
4	Save the color compensation data by naming the calibration file. ***Note:*** The file name must end in “.ccc”, e.g. data1.ccc.

Note: Once you have created and saved a color compensation calibration file, you can use that data to correct many future data sets (as long as you use the same combination of fluorophors).

Continued on next page

4.3.3. LCDA Front Screen: Using Color Compensation Files,
Continued

Using a Color Compensation File During Data Analysis

To use a calibration file to color compensate run data, follow the steps described below:

Step	Action
1	From the **Data Analysis Window**, open the file containing the run data (see Section 4.2 of this chapter). ***Result:*** The **LCDA Front Screen** will appear and display the run data.
2	Bracket the program segment to be analyzed in the **Temperature vs. Time** screen (see Section 4.3.1 of this chapter).
3	Under the **Color Compensation** menu, select „Load Calibration Data“, and click on the name of the stored **ccc** (calibration) file. ***Note:*** You may also click on the **Select CC Data** button (top center portion of screen) to find the stored **ccc** file.
4	To see what your data looks like after color compensation, do either of the following: • Click on the **Color Compensation** button (top center portion of screen), or • Select „Enable“ under the **Color Compensation** pull-down menu.
5	Click on the **Color Compensation** button again to return to the raw data.

4.4. LCDA Front Screen: Data Retrieval

Introduction

You can retrieve permanent copies of the data displayed on the LCDA Front Screen. This section describes options for data retrieval.

Print Data

You can print a hard copy of the data displayed on the screen.

To print either the upper or lower graph:

- From the **File** pull-down menu, select “Print Window”, and
- On the **Report** pull-down menu, highlight the graph to be printed. The Report function will launch the LightCycler Software report tool "Graphworks".

4.5. Further Analysis

Further Analysis of Amplification or Melting Curve Data

If you have collected data in specific formats, you can use the buttons at the top of the **LCDA Front Screen** to prepare them for further analysis, as follows:

If the data is displayed as...	**For further analysis, click...**	**And...**
Fluorescence vs**. Cycle** (e.g., fluorescence data that was acquired once per cycle)	**Quantification** button	Use Section 5 of this chapter to set up a Quantification analysis
Fluorsence vs**. Temperature** (e.g., fluorescence data acquired continuously from a melting experiment)	**Melting Curve** button	Use Section 6 of this chapter to set up a Melting Curve analysis

5. Display and Analysis of Quantification Data

5.1. Introduction

Quantification on the LightCycler

The LightCycler performs a quantification analysis by comparing the fluorescence of a PCR product of unknown concentration with the fluorescence of several dilutions of an external standard. For this analysis, the instrument considers only fluorescence values measured in the log-linear phase of amplification.

Note: To perform a quantification analysis the user must have defined at least two standards of known concentration in the **Loading Screen**. Ideally, standards are amplified under the same conditions as the unknowns and have the same amplification efficiency. We recommend using the same primer set to amplify both standards and unknowns.

Fluorescence Acquisition for Quantification Analysis

In order to quantify PCR product with the LightCycler, the instrument must be set up to acquire fluorescence once per cycle. This will generate a fluorescence curve that increases in value with each cycle as the product accumulates.

Use of Crossing Line to Quantify Unknowns

The most useful data occurs during the few cycles in which the fluorescence readings are above background and increase in a log-linear fashion. The LCDA quantification software is designed to generate a best-fit line from this log-linear region of each curve.

This best-fit line will be parallel to the x-axis and is called a **Crossing Line**. The point at which the **Crossing Line** intercepts the log-linear region of each fluorescence curve may be used to calculate the concentration of the sample that generated the fluorescence curve.

The instrument plots a standard curve of the **Crossing Line** intercepts of the standards vs. the known concentrations of these standards. By comparing the **Crossing Line** intercept of an unknown sample with the standard curve, the instrument generates a quantitative estimate of the starting copy number of the target DNA in the unknown.

5.2. Quantification: Selection of Analysis Method

Quantification Analysis Methods

Clicking on the **Quantification** button on the **LCDA Front Screen** will open the **Quantification Analysis** window. Once that window is open, the first step of the data analysis is to select the appropriate analysis method. There are two choices. The descriptions given below should help you determine the most suitable analysis.

Note: To choose either of the two methods, click on one of these buttons:

- **Fit Points**, or
- **Second Derivative Maximum**

Alternatively, you can choose either of the methods under "Analysis Method" in the **Quantification** menu. Note that the **Fit Points** option is called **Fit N Points Above Threshold** in this menu, while **Second Derivative Maximum** has the same name on both the menu and the button.

Fit Points Method (Default Method)

This method is possible with all applications. When quantitating samples containing low copy numbers of target DNA (typically less than 1000 copies), the **Fit Points** method often gives better results than **Second Derivative Maximum**. **Fit Points** also works well at high copy numbers, so if the template concentration in your samples ranges from low to high, you should use **Fit Points.** Additionally this method shows backward compatibility to the old software.

Note: Set up and analysis by the Fit Points method requires three steps: **Step 1: Baseline, Step 2: Noise Band** and **Step 3: Analysis.** These steps are described in detail in Sections 5.3–5.5 of this chapter.

Second Derivative Maximum Method

The **Second Derivative Maximum** method is better for analyzing high copy numbers (above 1000 copies per tube) since it requires no user input or bias. However, this method may have problems analyzing low copy numbers that lead to less well-behaved reactions.
The Second Derivative Maximum Method is a semiautomated method, that uses the shape of the curve to determine the target copy number. It is independent of user-borne influences and will produce identical data e.g. irrespective of baseline subtraction method.

Note: Set up and analysis by the Second Degree Maximum method requires only two steps: **Step 1: Baseline, Step 2: Analysis**. This method does not require setting a noise band (Step 2 under Fit Points above). See the next page for more information.

Continued on next page

5.2. Quantification: Selection of Analysis Method, Continued

Tabs on the Quantification Analysis Screen

The **Quantification Analysis** screen will display fluorescence curves for all the samples in the selected run.
If you chose the **Fit Points** method, you will see three tabs displayed at the top of the screen: **Step 1: Baseline, Step 2: Noise Band,** and **Step 3: Analysis**. ***Note:*** For more details on each stage, see the section of this chapter listed in the table below).
If you chose the **Second Derivative Maximum** method, you will see only two tabs: **Step 1: Baseline and Step 2: Analysis**.

To set up and perform the analysis, you must select each tab in turn, and define the appropriate data analysis parameters in the screen that appears. The following parameters must be defined:

Stage	Description	See Section
Step 1: Baseline	For valid comparison, all samples should have baseline fluorescence values close to zero. In this screen, you will define any **baseline corrections** that are necessary prior to analysis.	5.3
Step 2: Noise Band (Fit Points method only)	In this screen, you will set the noise band that defines **non-informative fluorescence data**.Typically, this is data that cannot be distinguished from the fluorescence noise in the earlier cycles of amplification. ***Note***: Not required for the **Second Derivative Maximum** method**. This method is a semi-automated approach to quantitative PCR. It solely interprets the shape of the curve to determine copy number. Consequently, it does not require setting a Noise Band, nor a Crossing Line. Also Baseline correction is solely performed to free the graph from effects of background variability. It will not have any effect on quantification as such.**	5.4
Step 3: Analysis	In this screen, you will determine the point of intersection between the fluorescence curves and a defined **crossing line**. You will select the type of threshold setting from several options. ***Note:*** The values for the crossing points are used to generate a standard curve which plots **Cycle Number vs. Log Concentration** and this standard curve will be used to calculate concentrations for unknown samples.	5.5

Continued on next page

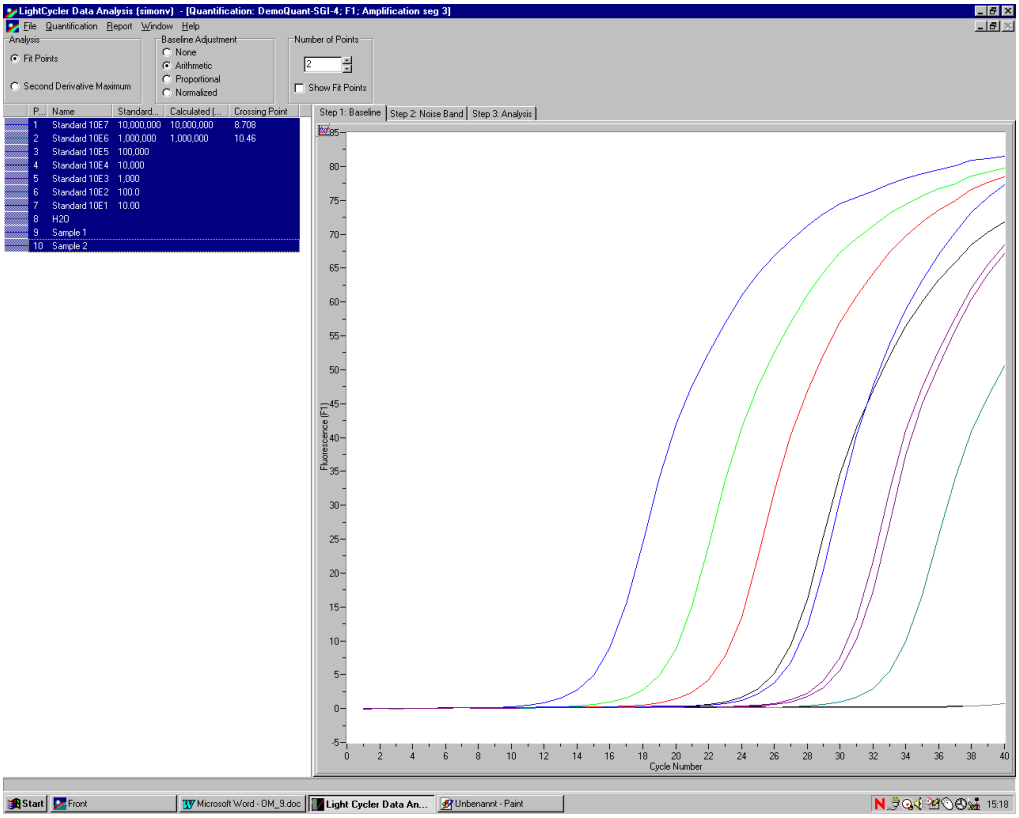
5.2. Quantification: Selection of Analysis Method, Continued

How to Use this Part of the Manual

Each of the steps in the table above are described more fully in Sections 5.2–5.5 of this chapter. You may use these sections to familiarize yourself with the steps of a Quantification Analysis.
After you are familiar with the required steps, use the convenient procedure (Section 5.6 of this chapter) to set up and perform Quantification Analysis.

5.3. Quantification Step 1: Baseline

Step 1: Baseline Screen



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5.3. Quantification Step 1: Baseline, Continued

Quantification Step 1: Baseline

Regardless of which analysis method you choose, you must use the **Baseline Screen** to define any baseline corrections necessary for data analysis.
Note: Baseline fluorescence may vary from sample to sample for several reasons including differences in sample preparation, differences in DNA content from tube to tube or pipetting error. However, in order to make sample to sample comparisons easier, you must set the baseline fluorescence for all samples to zero.
The **Fluorescence vs. Cycle Number** graph (shown on the previous page) has been carried over from the LCDA front screen and is used for baseline adjustment. As you select a method for baseline adjustment, this graph will change. The corrected graph will then be carried over to the next screen.

There are four options available for baseline adjustment:

Baseline adjustment	Description
None	No baseline adjustment.
Arithmetic (Default option)	Recommended for any application with high sample-to-sample background variation. In this method, the mean value of the five lowest measured data points for each sample will be calculated, then subtracted from each reading point in the sample. ***Note:*** This method is **best for SYBR Green I reactions,** since the template DNA will sometimes give a signal that is removed most efficiently by this method.
Proportional	When target DNA presents no baseline problem, the **Proportional** method can remove some of the tube-to-tube variations due to pipetting. ***Note:*** This method is **best for hybridization probes, TaqMan probes, Beacons,** and especially **suited for Dual Color experiments**.
Normalization	All fluorescence curves are displayed as a percent of maximum fluorescence. One hundred percent fluorescence is represented by the value 100. This brings all fluorescence curves to the same maximum value. When used with the Fit Points Method, it will alter the curves such, that incorrect quantification data may be produced. ***Note:*** This method is ***not*** **suited for analysis**.

Continued on next page

5.3. Quantification Step 1: Baseline, Continued

Sample Display

Samples are listed in a table to the left of the **Fluorescence vs. Cycle Number** graph in the order in which they were loaded. The table gives **display color, sample number, sample name**, known **concentration** (if entered prior to the run, e.g. for standards), **calculated concentration,** and **crossing point**.
The values in the last two columns will change as the criteria for analysis are further defined. Below the list of sample names are calculated values for the means and standard deviations of replicate samples.
***Note*: If all the described table columns are not visible, click on the left-hand boundary of the graph and drag it to the right to expose the data that lies behind it.**

Sample Selection

Select the samples to be displayed as follows:

- To display an individual sample, highlight it by clicking on the sample name.
- To display multiple samples, use shift-click.
- To selectively add or remove displayed samples, use control-click.

Change Graph Settings

To change any axis values of the graphs displayed click on the button on the top left corner of the graph.
This will bring up a **Customize Graph** screen. In this screen the user can define values for the axis which will then be carried into the LCDA display. Deselect the **Automatic Fit** option and enter values for the axis in the appropriate fields.

Data Retrieval

Any data displayed on the Baseline Screen can be retrieved as either a text report, a graphic or in spreadsheet format:

- From the **Report** pull-down menu, choose “Summary” option. This will generate a text report listing all the run parameters, all the analysis parameters, and all sample names and concentrations.
- To obtain a hard copy of one of the displayed graphs, select one of the following from the **Report** menu: the “Baseline Adjustment“ graph from **Step 1**, the “Noise Band“ graph from **Step 2**, the “Analysis“ from **Step 3**, and the “Standard Curve“ from **Step 3**.
- To obtain the data in a tab-delimited spreadsheet format select “Export“ from the **Quantification** menu. This will create a text file that can be opened in any spreadsheet program.

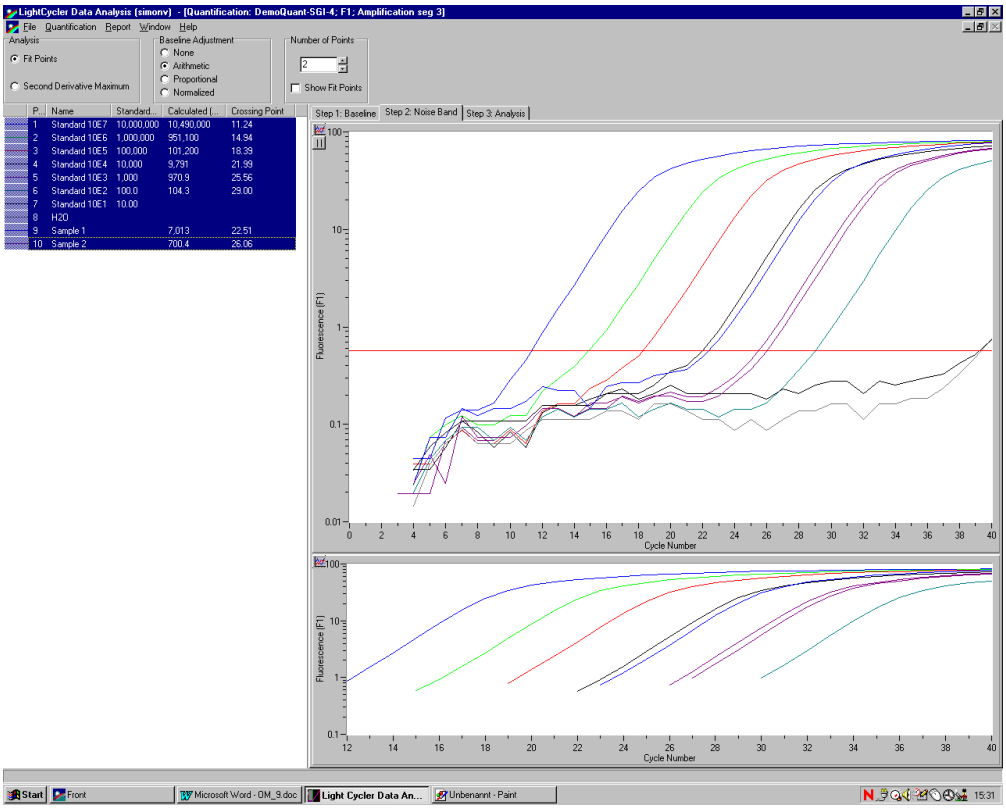
5.4. Quantification Step 2: Noise Band

Introduction

The **Noise Band** is used to define fluorescence data that is non-informative. Such data typically includes those data points in the earlier part of the amplification reactions that cannot be distinguished from the fluorescence ”noise”.

***Note*: If you are using the Second Derivative Maximum methods, skip this step. Quantification analysis with the Second Derivative Maximum method does not require manual setting of a Noise Band. Consequently this tab is not accessible, when Second Derivative Maximum method is selected.**

Step 2: Noise Band Screen



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5.4. Quantification Step 2: Noise Band, Continued

Setting the Noise Band

Both graphs on the **Noise Band Screen** show fluorescence values on a log scale, **Log Fluorescence vs. Cycle Number.**
A red **Noise Band** appears on the upper graph. You will move this band up or down to define which data points will be included in the analysis. All data points that fall below the **Noise Band** will be excluded. The data selected for analysis (i.e., above the **Noise Band**) will be displayed in the lower graph.
Note: As a result of the baseline correction, some data points may be corrected to a value below zero. These data points cannot be displayed in a log scale. As a result some lines are interrupted, in the noise band graph. This has no effect on correctness of calculated data.

Sample Display

Select the samples to be displayed as follows:

- To display an individual sample, highlight it by clicking on the sample name.
- To display multiple samples, use shift-click.
- To selectively add or remove displayed samples, use control-click.

***Note*: If all the table columns are not visible, click on the left-hand boundary of the graph and drag it to the right to expose the data that lies behind it.**

Change Graph Settings

To change any axis values of the graphs displayed click on the button on the top left corner of the graph.
This will bring up a **Customize Graph** screen. In this screen the user can define values for the axis which will then be carried into the LCDA display. Deselect the **Automatic Fit** option and enter values for the axis in the appropriate fields.

Data Retrieval

Any data displayed on the Noise Band Screen can be retrieved as either a text report, a graphic, or in spreadsheet format:

- From the **Report** pull-down menu, choose "Summary" option. This will generate a text report listing all the run parameters, all the analysis parameters, and all sample names and concentrations.
- To obtain a hard copy of one of the displayed graphs, select one of the following from the **Report** menu: the "Baseline Adjustment" graph from **Step 1**, the "Noise Band" graph from **Step 2**, the "Analysis" from **Step 3**, and the "Standard Curve" from **Step 3**.
- To obtain the data in a tab-delimited spreadsheet format select "Export" from the **Quantification** menu. This will create a text file that can be opened in any spreadsheet program.

5.5. Quantification Step 3: Analysis

Introduction

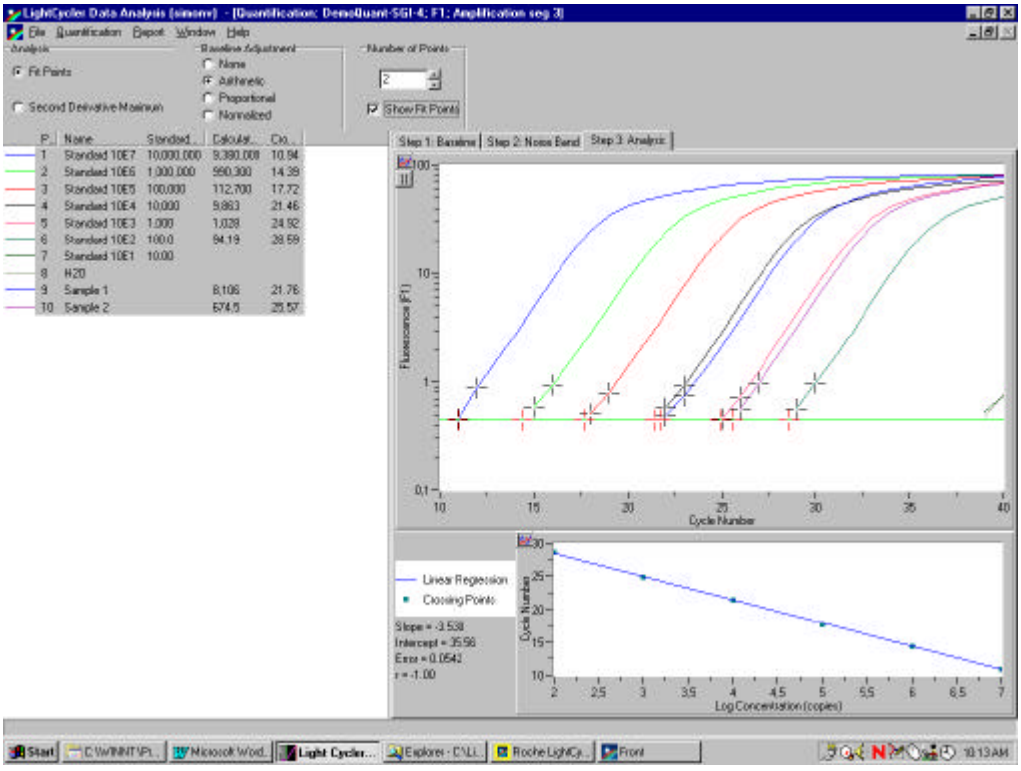
Once background fluorescence has been defined, the fluorescence data can be used for quantification in the **Analysis Screen**. The most informative data lies in the log-linear portion of the fluorescence curve and starts at the cycle number where fluorescence rises above background.
The LCDA software uses the intersection between each fluorescence curve and a **Crossing Line, set to the level of the Noise Band** to give a value for a **Crossing Point**.

The **Crossing Point** values for a set of standards, expressed as a fractional cycle number, can be plotted against **log concentration** to give a standard curve. You can use this standard curve to determine the starting template concentration of unknown samples.

Note:

- For the **Fit Points** analysis method, the first step of the data analysis requires setting a **Crossing Line**.
- For the **Second Derivative Maximum** method, the **Crossing Points** are defined automatically, so no **Crossing Line** needs to be set.

Step 3: Analysis Screen, Fit Points Method



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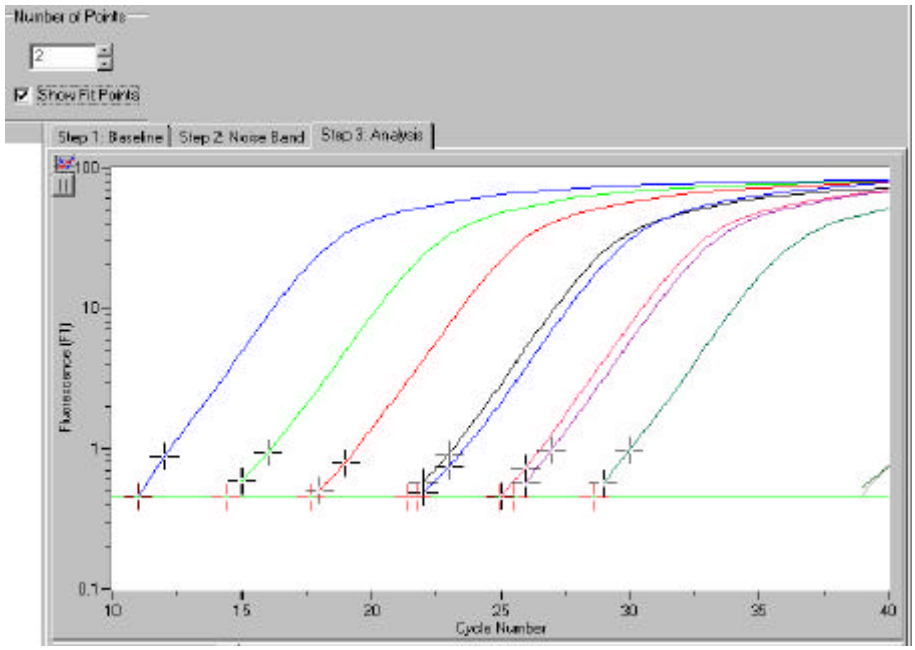
5.5. Quantification Step 3: Analysis, Continued

Fit Points Analysis Method: Crossing Line and Number of Points

In the **Fit Points** method, you will need to:

1) Position a green **Crossing Line** in the graph (by dragging it with the mouse pointer). The automatic default position of the **Crossing Line** is set on the Noise Band. However, this setting must not be optimal.
2) Tell the instrument the **Number of Points** to be selected. This value is your best estimate of how many meaningful fluorescence values (i.e., fluorescence values above the Noise Band) were acquired while fluorescence was increasing in a log-linear fashion. Enter this estimate in the **Number of Points** box.
   Note: **The default Number of Points value is 2 points. In most cases, this is the best number to use for reliable analysis.**



5.5. Quantification Step 3: Analysis, Continued

Fit Points Method: Crossing Points

The software uses the selected **Number of Points** to calculate a "best fit" log-linear fluorescence curve for each sample. The intersection (marked with a red cross) of this line with the **Crossing Line** is the so-called **Crossing Point** (expressed as a fractional cycle number, i.e. a cycle number that is calculated to two decimal places).

This **Crossing Point** value is displayed in the table column immediately to the left of the graph. All the **Crossing Points** of the standards are used to plot a graph of **Cycle Number vs. Log Concentration.** This standard curve, in turn, is used to estimate the concentration of each sample (see below).

P...	Name	Standard...	Calculated (copies)	Crossing Point
1	Standard 10E7	10,000,000	10,230,000	11.51
2	Standard 10E6	1,000,000	939,000	15.19
3	Standard 10E5	100,000	107,600	18.52
4	Standard 10E4	10,000	9,598	22.24
5	Standard 10E3	1,000	999.7	25.72
6	Standard 10E2	100.0	100.8	29.24
7	Standard 10E1	10.00		
8	H2O			
9	Sample 1		7,287	22.66
10	Sample 2		715.2	26.23

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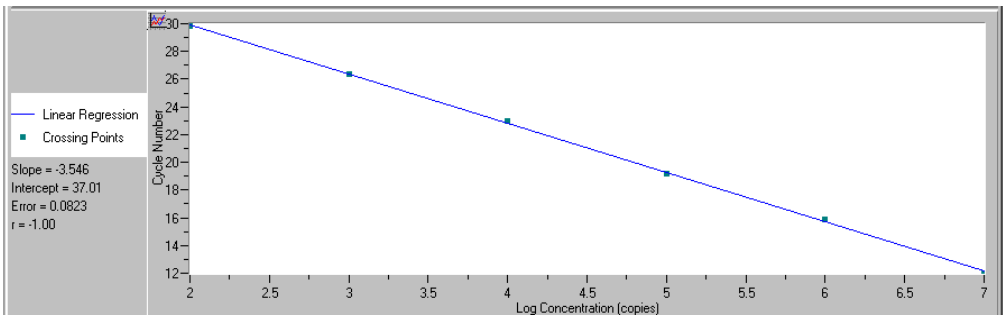
5.5. Quantification Step 3: Analysis, Continued

Cycle Number vs. Log Concentration

For each standard sample (as designated in the Loading Screen during the Programming phase), the instrument plots the Crossing Point against the known concentration of the standard. The resulting standard curve is shown as a graph of **Cycle Number vs Log Concentration** in the window at the bottom of the **Analysis Screen**.



Linear regression analysis is used to generate the straight line plot shown in the window (blue line). Values for the **slope** of the line, the Y **intercept,** the mean squared **error** of the determination and "r", the coefficient of correlation are displayed in the box to the left of the curve.

The standard curve is then used to convert the Crossing Point value of an unknown sample to a calculated sample template concentration. This concentration value is displayed in the "Calculated Concentration" column of the sample table (left portion of screen).

Setting the Crossing Line

The automatic default position of the **Crossing Line** is set on the Noise Band. However, this setting must not be optimal.

Note: For optimal results, you may use the mouse to move the **Crossing Line** (upper graph) away from its default value. Adjust the **Crossing Line** so that the **Error** value (displayed to the left of the lower graph) is at its lowest value and the "**r**" value **(**correlation coefficient**)** is closest to one. As you move the Crossing Line, you should also monitor the calculated standard values in the sample table; at the optimal setting for the line, the calculated values of the standards should be closest to their known values.

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5.5. Quantification Step 3: Analysis, Continued

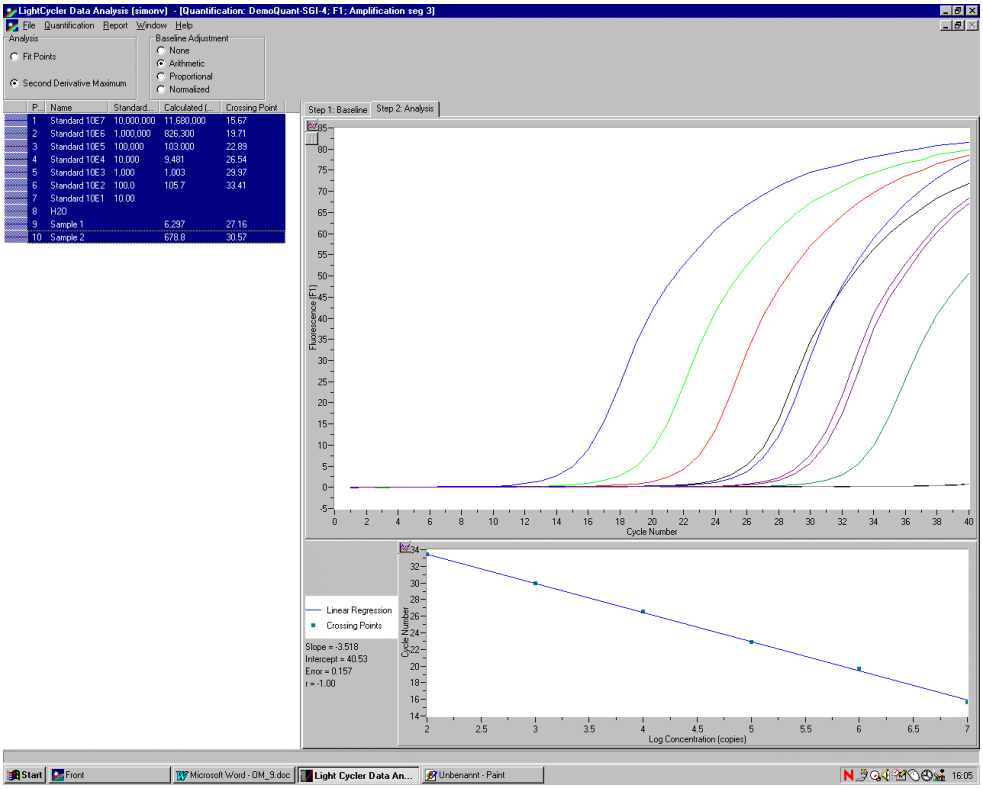
Analysis: Second Derivative Maximum Analysis Method

The **Second Derivative Maximum** method automatically calculates the fractional cycle number of the **Crossing Point** (see previous page).

The **Second Derivative Maximum** is the point at which the rate of change of fluorescence is fastest. It usually occurs in the cycle where the sample fluorescence can first be distinguished from background fluorescence.

The **Second Derivative Maximum** can be used as an accurate predictor of concentration. The fractional cycle number at which the **Second Derivative Maximum** occurs for each sample is indicated in the"Crossing Point" column immediately to the left of the graph. The values in this column are used to plot **Cycle Number vs. Log Concentration** (below) for the standards. This standard curve, in turn, is used to estimate the concentration of each sample.

***Note*: Unlike the Fit Points method, the Second Derivative Maximum method does not require you to define the log-linear segment of each curve, nor does it require you to set a Noise Band.**



5.5. Quantification Step 3: Analysis, Continued

Sample Table

The results of the quantification analysis are displayed in the sample table at the top left corner of the screen. Samples are listed in the order in which they were loaded, with **display color** followed by **sample number**, **sample name,** the known concentration of the **standard** (if entered prior to the run), the **calculated concentration,** and the calculated **crossing point**.

***Note*: If all the table columns are not visible, click on the left-hand boundary of the graph and drag it to the right to expose the data that lies behind it.**

Change Graph Settings

To change any axis values of the graphs displayed click on the button on the top left corner of the graph.
This will bring up a **Customize Graph** screen. In this screen the user can define values for the axis which will then be carried into the LCDA display. Deselect the **Automatic Fit** option and enter values for the axis in the appropriate fields.

Data Retrieval

Any data displayed on the Analysis Screen can be retrieved as either a text report, a graphic, or in spreadsheet format:

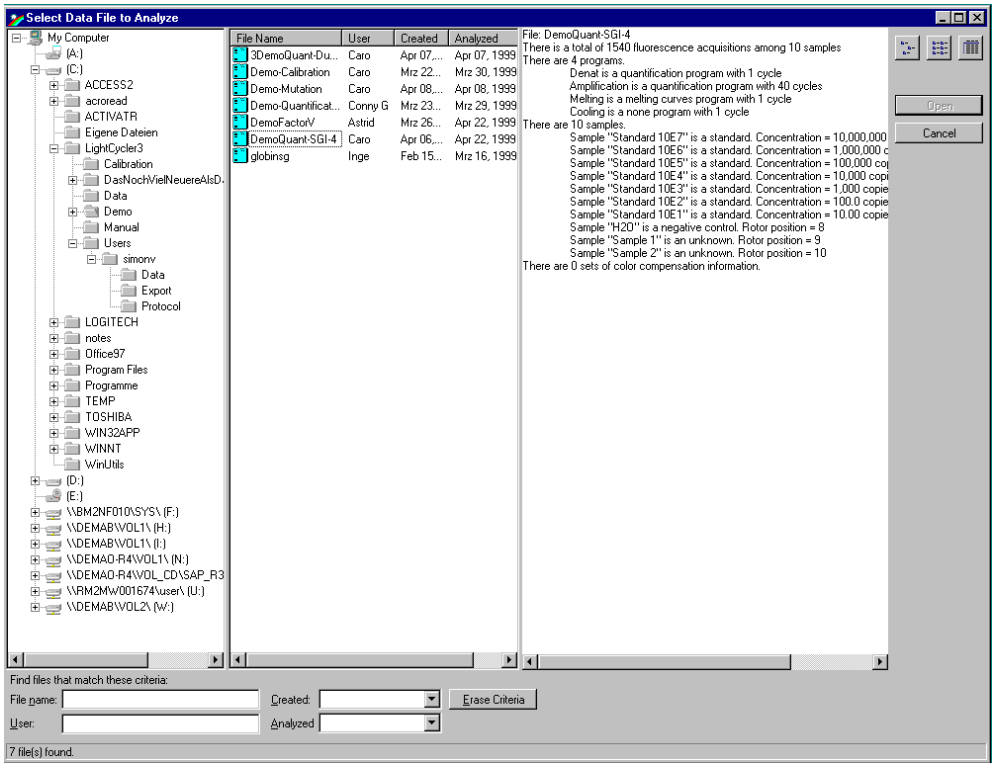
- From the **Report** pull-down menu, choose "Summary" option. This will generate a text report listing all the run parameters, all the analysis parameters, and all sample names and concentrations.
- To obtain a hard copy of one of the displayed graphs, select one of the following from the **Report** menu: the "Baseline Adjustment" graph from **Step 1**, the "Noise Band" graph from **Step 2**, the "Analysis" from **Step 3**, and the "Standard Curve" from **Step 3**.
- To obtain the data in a tab-delimited spreadsheet format select "Export" from the **Quantification** menu. This will create a text file that can be opened in any spreadsheet program.

5.6. A Procedure for Analyzing Quantification Data

Quantification Analysis

Use the procedure below to set up and perform quantification analysis on fluorescence data.

Note: Data for this analysis should have been acquired (measured) once per cycle.

Step	Action
1	• Click the **Data Analysis** button on the **LightCycler Front Screen** (or choose "LCDA" from the **Analysis** menu). • Open the **Data** file to be analyzed. ***Remember:*** Every user has an individual data folder. You can see only data files belonging to your user name. ***Result:*** The data file you select will open and be displayed on the **LCDA Front Screen.** 

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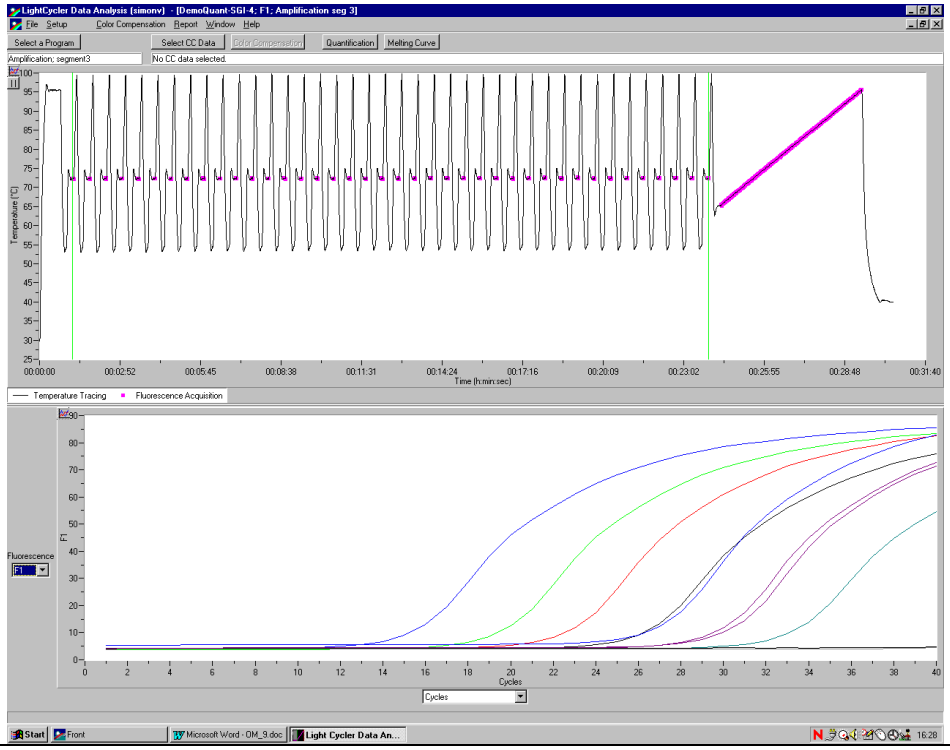
Quantification Analysis (continued)

Step	Action

2	In the upper **Temperature vs. Time** graph, define the amplification segment of the Experimental Protocol. You may do this in either of two ways:
	
	• Define the segment automatically, as follows.
	  • Click on the **Select a Program** button at the top left of the screen.
	  • Choose "Quantification Program" to automatically make the cursors bracket the amplification segment on the upper graph.
	  Result: The x-axis in the lower graph switches automatically to **Cycles.**
	
	• Define the segment manually, as follows:
	  • Drag the green cursors until they bracket the amplification segment on the upper graph.
	  • Select **Cycles** as the x-axis scale for the lower graph (in the pull-down menu below the graph).
	
	 

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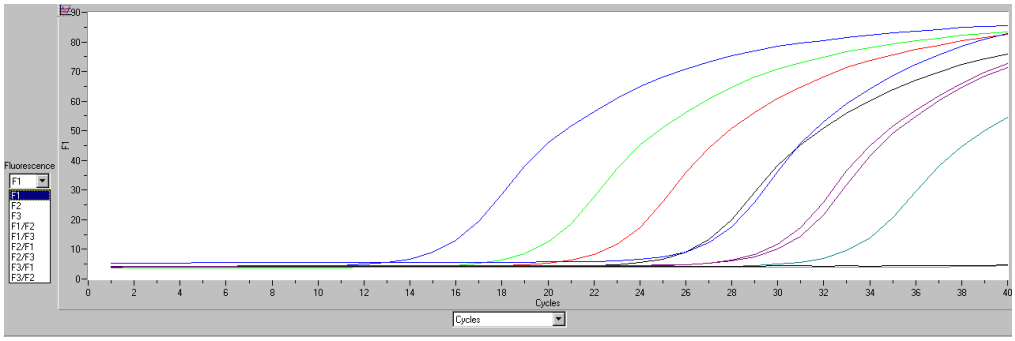
5.6. A Procedure for Analyzing Quantification Data, Continued

Quantification Analysis (continued)

Step	Action
3	In the pull-down menu to the left of the lower graph, select the fluorescence display method appropriate for your data. ***Examples:*** Choose F1 for SYBR Green I, F2 for hybridization probes with an LC-Red 640 label, and F3 for hybridization probes with an LC-Red 705 label. 
4	• If color compensation is required, click on the **Select CC Data** button (at top of screen) and click on the **ccc data file** to be used. ***Note:*** You can also choose "Load Calibration" under the **Color Compensation** pull-down menu and select the **ccc** file to be used. • Click on the **Color Compensation** button (next to the **Select CC Data** button) to activate the color compensation software. ***Note:*** When compensation is active, the name of the **ccc** file appears after the name of the experimental analysis (in the main bar at the top of the screen).

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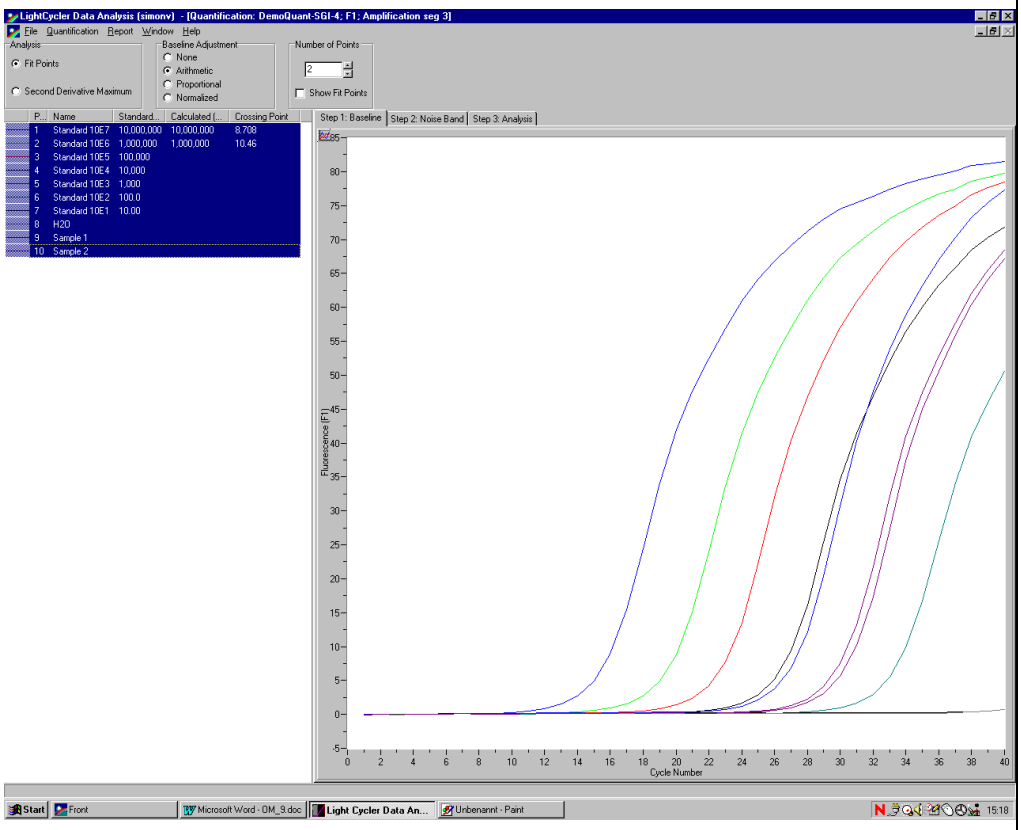
5.6. A Procedure for Analyzing Quantification Data, Continued

Quantification Analysis (continued)

Step	Action
5	Click on the **Quantification** button at the top of the screen. ***Note:*** You may also choose "Quantification Analysis" in the **Setup** menu. ***Result:*** The quantification analysis software will be activated and the **Step 1: (Baseline) Screen** will appear. 

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Quantification Analysis (continued)

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>6</td><td>Choose the quantification method you want by clicking on the appropriate name in the Analysis box (top left corner of screen; see the picture in Step 5 above).

There are two available methods, which are described briefly below:

<table>
<tr><th>Method</th><th>Suitable for analysis when</th></tr>
<tr><td>Fit Points
(default; 3-step manual calculation method)</td><td>• Possible with all applications.
• The starting target concentration is below 1000 copies/sample
• Template concentrations in samples range from very low to very high
• Backward compatibility to old software</td></tr>
<tr><td>Second Derivative Maximum
(2-step semiautomated calculation method)</td><td>• "Quick glance" with a data driven algorithm.
• The starting target concentration is high (typically above 1000 copies/sample
<u>Note:</u> Not recommended for analysis of low copy numbers</td></tr>
</table>

<u>Note:</u> For more information on the two methods, see Section 5.2 of this chapter.</td></tr>
</table>

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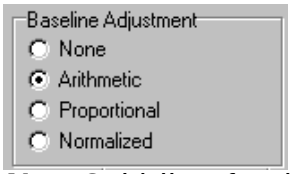
Quantification Analysis (continued)

Step	Action
7	• In the sample table, highlight all the samples to be analyzed. • Select a baseline subtraction method from the options under **Baseline Adjustment** at the top of the screen:  ***Note:*** Guidelines for choosing the appropriate baseline are given below:

Baseline adjustment	Description
None	No baseline adjustment.
Arithmetic (Default option)	Recommended for any application with high sample-to-sample background variation. In this method, the mean value of the five lowest measured data points for each sample will be calculated, then subtracted from each reading point in the sample. ***Note:*** This method is **best for SYBR Green I reactions,** since the template DNA will sometimes give a signal that is removed most efficiently by this method.
Proportional	When target DNA presents no baseline problem, the **Proportional** method can remove some of the tube-to-tube variations due to pipetting. ***Note:*** This method is **best for hybridization probes, TaqMan probes, Beacons,** and especially **suited for Dual Color experiments**.
Normalization	All fluorescence curves are displayed as a percent of maximum fluorescence. One hundred percent fluorescence is represented by the value 100. This brings all fluorescence curves to the same maximum value. When used with the Fit Points Method, it will alter the curves such, that incorrect quantification data may be produced. ***Note:*** This method is ***not*** **suited for analysis**.

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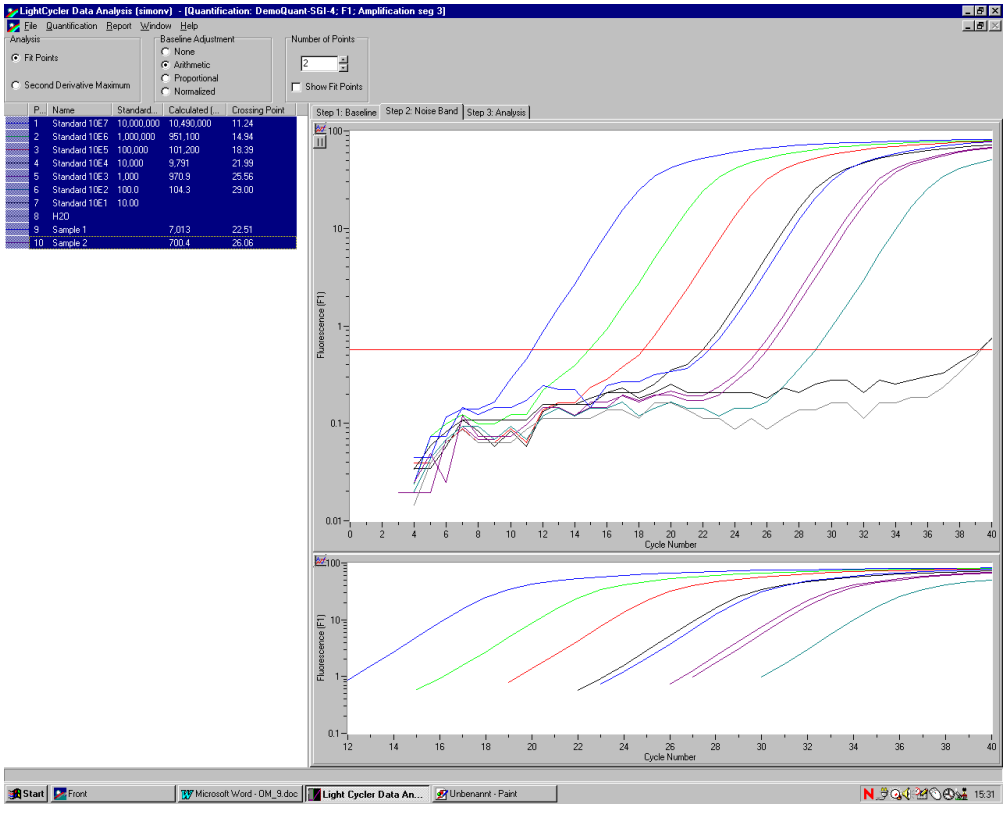
5.6. A Procedure for Analyzing Quantification Data, Continued

Quantification Analysis (continued)

Step	Action
8	Depending upon the quantification method you chose in Step 6, do one of the following: • If you chose the **Fit Points** method, go to **Step 9** below. • If you chose the **Second Derivative Maximum** method, skip Steps 9–12 and go to **Step 13**.
9 (for Fit Points Method only)	Click the **Step 2: Noise Band** tab at the top of the screen. ***Result:*** The **Noise Band Screen** appears. In this screen you will see two graphs of **Log Fluorescence** vs. **Cycle number**. You will notice a red horizontal line in the top graph. This is the **Noise Band**. 

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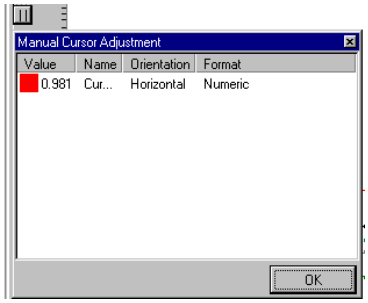
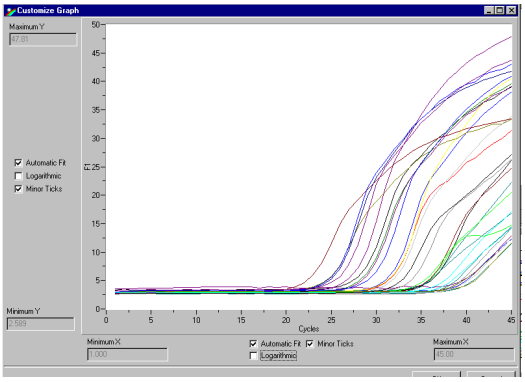
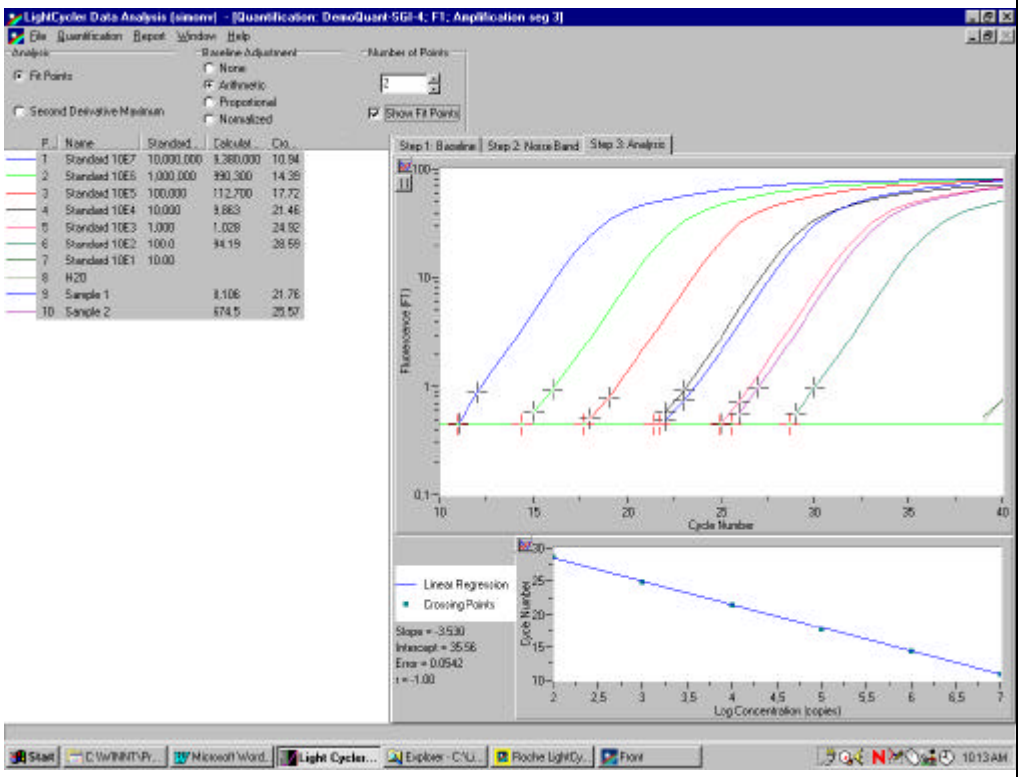
Quantification Analysis (continued)

Step	Action
10 Cont.	In the top graph (see picture in Step 9 above), move the red **Noise Band** to a point just above the baseline noise. ***Note:*** You may also set the **Noise Band** by clicking the button under the icon (at the top left corner of the graph). In the window that opens, specify the cursor position by typing the appropriate value.  ***Tip:*** You may need to enlarge the graph to set the Noise Band properly. To enlarge the graph, click on the gray bar separating the two graphs and drag it down. After the noise band has been set, you may make the graph smaller.
11 (for Fit Points Method only)	To change any axis values of the graphs displayed click on the button on the top left corner of the graph. This will bring up a **Customize Graph** screen. In this screen the user can define values for the axis which will then be carried into the LCDA display. Deselect the Automatic Fit option and enter values for the axis in the appropriate fields.  In the window that opens, you may change the values at the x- and y-axis, or choose an **Automatic Fit** or **Logarithmic** display. Click on the Minor **Ticks** box to finely adjust the scaling of the x- or y-axis.

Continued on next page

5.6. A Procedure for Analyzing Quantification Data, Continued

Quantification Analysis (continued)

Step	Action
12 (for Fit Points Method only)	As you move the **Noise Band** on the upper graph**,** look at the lower graph of **Log Fluorescence** vs. **Cycle Number.** This graph displays only the data above the noise band. After you set the Noise Band, this lower graph should show only the log-linear and plateau phases of amplification for each sample selected. You should not see any background fluorescence on this graph. ***Tip:*** If you still see background fluorescence in the lower graph, move the Noise Band to a higher level in the upper graph.
13	• Click the **Analysis** tab (labeled either **Step 3** or **Step 2**, depending on the method chosen) at the top of the screen. ***Result:*** The **Analysis Screen** appears. In this screen you will see a graph of **Log Fluorescence** vs. **Cycle Number.** This (upper) graph has been carried over from the previous screen. The lower graph is a calculated standard curve, which will be used to determine the concentration of the unknowns. 

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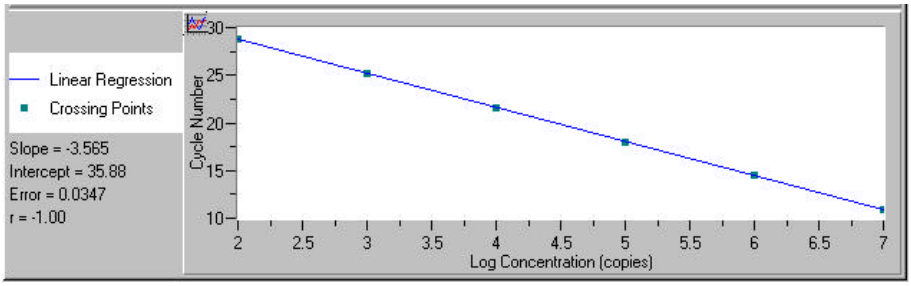
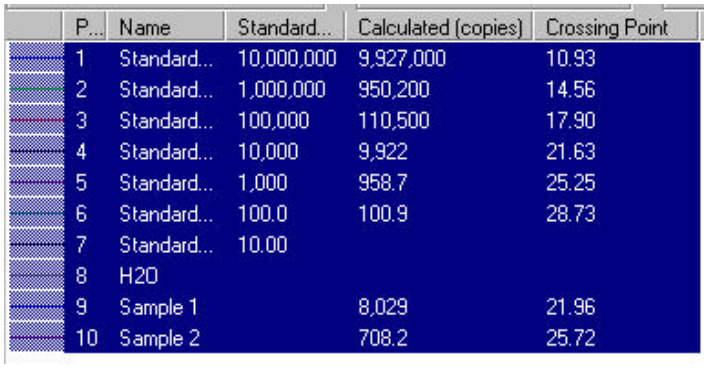
5.6. A Procedure for Analyzing Quantification Data, Continued

Quantification Analysis (continued)

Step	Action
14	Do one of the following: • If you chose the Fit Points method, go to Step 15. ***Note:*** You will notice a green **Crossing Line** on the top graph (see picture in Step 13). You will use this line to define the **Crossing Points** in Step 16. • If you chose the Second Derivative Maximum method, skip Steps 15 and 16 and go to Step 17. ***Note:*** In this method, the **Crossing Points** are automatically calculated. The method does not require the setting of a Crossing Line, so the line does not appear on the top graph (see Section 5.5 for more information).
15 (Fit Points Method only**)**	• Click on the **Show Fit Points** box (below the **Number of Points** window) to display the number of fluorescent readings that will be used to generate the fluorescence curve for each sample. • To change the default value (2 points), type a new value in the box. ***Caution:*** Select only those points that were acquired while the reaction was in the log-linear phase. ***Note:*** In most cases, the default value produces a reliable analysis. For details on the **Number of Points** setting, see Section 5.5 in this chapter.
16 (Fit Points Method only	The instrument will set a best-fit **Crossing Line** automatically, but that line can be moved manually up and down between the Fit Points in the log-linear phase. As it moves, the statistical analysis of the standard curve (lower graph shown in the photo below) will change (see also Section 5.5). Use the mouse to drag the **Crossing Line** until the **Error** value (box at left side of photo below) is at its lowest value and the **"r"** value (correlation coefficient) is closest to one. As you move the line you should also monitor the calculated standard values in the sample table; at the optimal setting, the calculated values for the standards should be closest to their actual values. 

Continued on next page

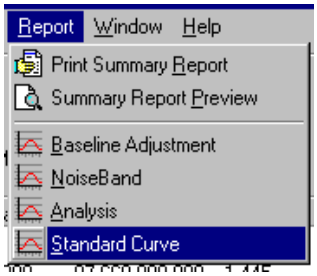
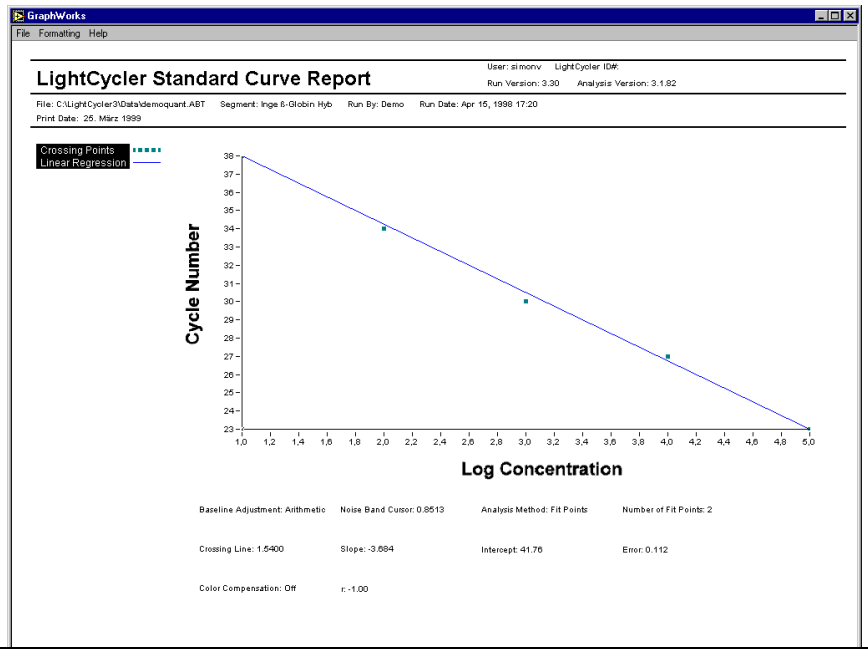
Quantification Analysis (continued)

Step	Action
17	Read the calculated concentrations of the unknowns from the columns of the sample table (to the left of the graphs). ***Note:*** If all the columns of the sample table are not visible, click on the left-hand boundary of the graph and drag it to the right to expose the data that lies behind it.  ***Note:*** For both quantification methods, the **Crossing Points** are used to create the standard curve of **Cycle Number** vs. **Log Concentration** (lower graph shown in Step 13). The instrument uses the standard curve to calculate the concentration of the unknowns shown in the sample table.

	P...	Name	Standard...	Calculated (copies)	Crossing Point
	1	Standard...	10,000,000	9,927,000	10.93
	2	Standard...	1,000,000	950,200	14.56
	3	Standard...	100,000	110,500	17.90
	4	Standard...	10,000	9,922	21.63
	5	Standard...	1,000	958.7	25.25
	6	Standard...	100.0	100.9	28.73
	7	Standard...	10.00		
	8	H2O			
	9	Sample 1		8,029	21.96
	10	Sample 2		708.2	25.72

Continued on next page

Quantification Analysis (continued)

Step	Action
18	From the **Report** pull-down menu, choose the **"**Summary" option. This will generate a text report (shown below) listing all the run parameters, analysis parameters, sample names and concentrations: • For a hard copy of the data, select "Print Window" from the pull-down **File** menu. • For a tab-delimited text file of the data, which may be opened in any spreadsheet program, select "Export" from the pull-down **Quantification** menu.
	• To obtain a hard copy of one of the displayed graphs, choose the appropriate graph in the **Report** pull-down menu (below) and select "Print Window" from the **File** menu  

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5.6. A Procedure for Analyzing Quantification Data, Continued

Quantification Analysis (continued)

Step	Action
19	To end the quantification analysis, select "Close the Window" from the **Window** menu at the top of the screen. ***Result:*** The software will display a window containing the original data for the run. This allows you to define and analyze any melting curve data acquired during the run (as detailed in Section 6 of this chapter).

6. Display and Analysis of Melting Curve Data

6.1. Introduction

Melting Curve Analysis on the LightCycler

The LightCycler obtains a melting curve by monitoring the denaturation profile of a PCR product in the prescence of either SYBR Green I or hybridization probes. Melting curves can be used to determine the melting temperature of the amplification products. Since the melting temperature of a DNA product is characteristic of its GC content, length and sequence, melting temperature can be used as a form of product identification.

The LightCycler analysis software automatically differentiates the melting curve data and presents it as melting peaks.

In addition, the melting profile of a sequence-specific hybridization probe can be used for rapid genotyping and mutation detection.

Setting up a Melting Curve Analysis

We recommend that an additional post-amplification melt cycle be included in all LightCycler protocols that use SYBR Green I as detection format.

The melt cycle must be included in the Experimental Protocol at the end of a run. Typically, a melt program is linked to a cycling program. It contains three steps: a rapid denaturation step, a short hold at an optimum annealing temperature, and a slow denaturation step.

During the final denaturation step, fluorescence should be acquired (measured) continuously to generate a high-resolution melting curve for every sample.

Beginning Data Analysis of a Melting Curve

To analyze melting curves, you must first display the run data graphically as **Fluorescence vs. Temperature** in the **LCDA Front Screen**.

In the **Temperature vs. Time** graph at the top of the **LCDA Front Screen**, you will move the two cursors so they bracket the segment representing the melt.

Note: It is possible to analyze only part of a melt by bracketing only that part with the cursors. You should do this in the **LCDA Front Screen**, since there will be no other opportunity to change the selected temperature segment during the analysis.

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Tabs on the Melting Curve Analysis Screen

After you click on the **Melting Curve** button on the LCDA Front Screen, the **Melting Curve Analysis** screen will appear and display fluorescence curves for all the samples in the selected run.

You will see three tabs displayed at the top of the screen, one for each stage of the melting curve analysis (shown in the table below).
Note: For more details on each stage, see the section of this chapter listed in the table.
To set up and perform the analyss, you must select each tab in turn, and define the appropriate analysis parameters in the screen that appears. The following parameters must be defined:

Stage	Description	See Section
Step 1: Melting Peaks	In this screen, you must select the calculation method used to generate melting peaks from the raw fluorescence data. Calculation options include linear fit of the data, polynomial fit of the data; and a background correction method that removes non-specific fluorescence	6.2
Step 2: Peak Areas	In this screen, you use the analysis software to calculate the area under each melting peak. ***Note:*** This type of analysis is necessary if the peak area is to be used to measure product concentration.	6.3
Step 3: Extra: Manual Tm	In this screen, you use the software to determine melting temperature (T_m) of any product to a fraction of a degree. You will manually move any of four cursors until it sits on each melting peak you wish to analyze. ***Note:*** The melting temperature of the product is the highest point of that melting peak.	6.4

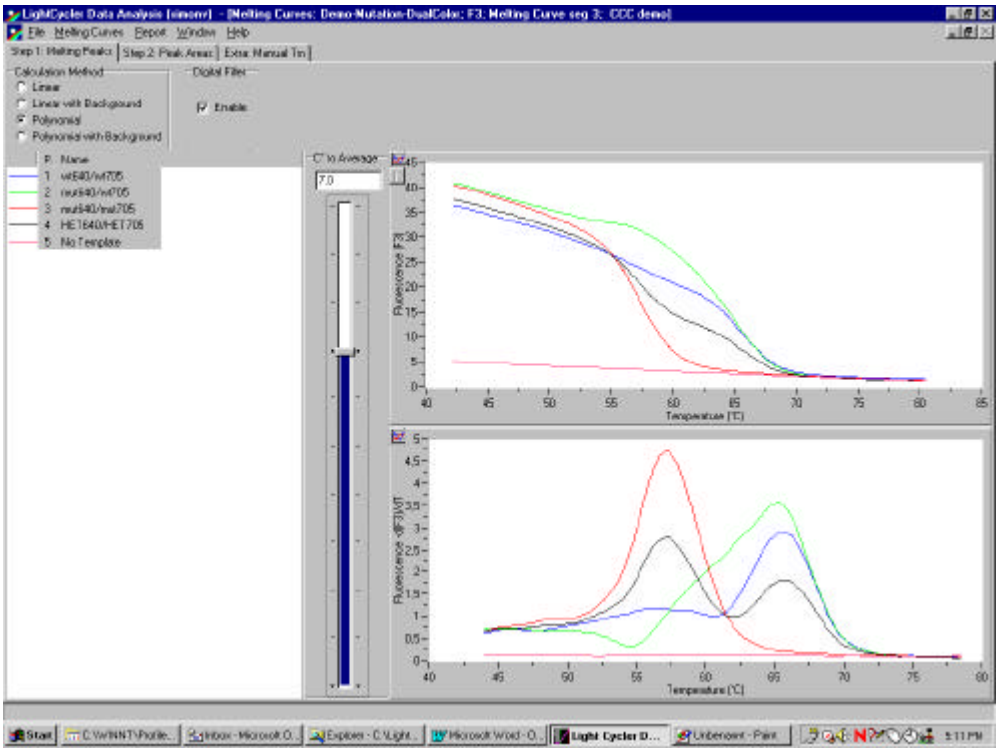
How to Use this Part of the Manual

Each of the steps in the table above are described more fully in Sections 6.2–6.4 of this chapter. You may use these sections to familiarize yourself with the steps of a Melting Curve Analysis.
After you are familiar with the required steps, use the convenient procedure (Section 6.5 of this chapter) to set up and perform Melting Curve Analysis.

6.2. Melting Curve Step 1: Melting Peaks

Introduction

The **Melting Peaks Screen** displays two graphs. The upper graph, which is carried over from the LCDA Front Screen, shows run data as **Temperature vs. Fluorescence.** The calculated first derivative of the run data is shown in the lower graph as **Temperature vs. –dF/dT**.
Note: The data in the lower graph displays "melting peaks" for each of the amplified products.



The screen lists several options that affect the way the data in the lower graph is presented. The available options are:

- Calculation method (box in the left corner of screen)
- °C to average (slider in middle of screen)
- Digital filter (check box in upper left portion of screen)

Note: The function of each of these options is described in detail in the next few pages.

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Melting Curve Calculation Methods

You may choose any of four calculation methods to generate melting peaks from the raw fluorescence data.

Method	Description
Linear	This method just serves for backward compatibility. It correspond to melting curve calculation method in the software version 1.2. This method does not produce a derivative estimate as accurate as the polynomial fit. ***Note:*** For a quick demonstration of the differences between linear and polynomial fit, look at the melting peaks calculated with each method as you increase the **°C to Average** slider towards its upper limit of 10°C. While the polynomial method peaks have some artifacts, they will still be usable. The linear method peaks will be unrecognizable in many instances.
Polynomial (default option)	This calculation method is the more robust method than the linear fit and thus recommended for all cases of Melting Curve Analysis.
Linear with Background or **Polynomial with Background**	**With Background** is an "enhancement" of the first two methods. Background subtraction compensates for the non-specific effects of temperature on fluorescence. ***Note:*** Background subtraction is especially important for the calculation peak areas during Melting Curve analysis.

Recommended Calculation Method

For most analyses, we recommend choosing **Polynomial with Background.**

°C to Average

The slider labeled **°C to Average** (near center of screen) sets the temperature interval used to calculate the derivative data in the lower graph. The default value is 2.0°, which works well with most applications. However, you can use the slider to increase (up to 10°).

Note: The optimum number of °**C to average** depends on the calculation method selected to obtain the derivative data. If you choose a **Polynomial** calculation method, the software automatically resets **°C to average** to a higher value (4°) than the default 2° used with a **Linear** calculation method.

Continued on next page

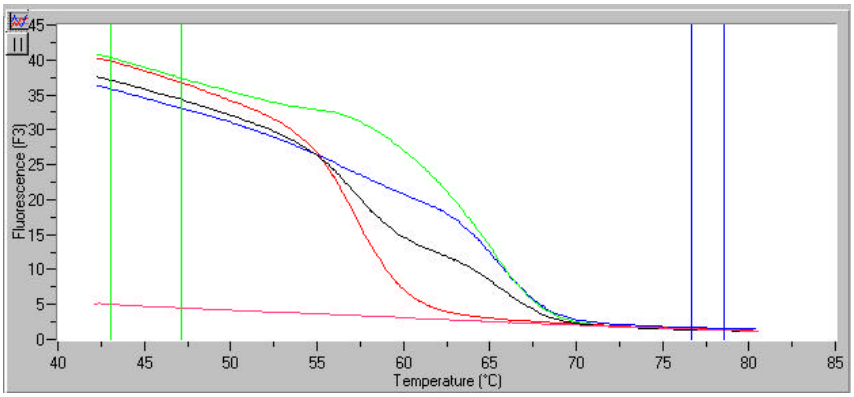
Digital Filter

When enabled, the digital filter damps out the background noise with a DISPO (digital smoothing polynomial) filter.
Recommendation: **Almost always enable this filter (that is, check the Enable box).**
Exception: Do not enable the filter when analyzing melting curves with a lot more data points (e.g. few samples monitored continuously) or a lot fewer data points (e.g. data acquired only once per °C) than a typical melting curve (e.g., melting curve analysis of a full rotor of samples, with 0.1°C stepwise acquisition).
Another exception is a Melting Curve Analysis with three or less samples. In this case, the digital filter may not be able to produce a correct graph and thus should be disabled.

Cursor Setting for Background

Background subtraction serves to remove the nonspecific effects of temperature on fluorescence.
If you select a calculation method with background subtraction, four vertical cursors will appear on the **Temperature vs. Fluorescence** graph:

- **End Cursors (blue):** Move these cursors into the flat fluorescence region that occured after all DNA melted. These cursors determine the final fluorescence background.
  Note: The distance between the cursors is not important.
- **Beginning Cursors (green):** Move these cursors into the fluorescence region that was measured before any product melted. These cursors determine the pure effect of temperature on the fluorescent signal.
  Note: The distance between the cursors should be as wide as possible without including any melt data.
  Caution: Do not include rising regions of the curve in which annealing was still taking place.



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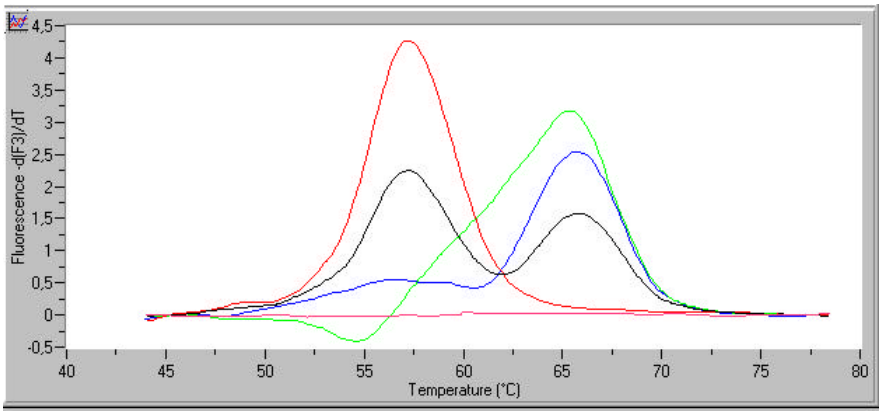
6.2. Melting Curve Step 1: Melting Peaks, Continued

Temperature vs. –dF/dT

The lower graph displays the negative first derivative of the melting curve data in the upper graph. It shows melting peaks for each sample.
Note: The melting temperature (top of a peak) is be used to identify the product.



Sample Display

Samples are listed in a table to the left of the **Fluorescence vs. Temperature** graph in the order in which they were loaded. The table gives sample number, display color, and sample name.
Tip: If you do not see all the table columns, click on the left-hand boundary of the graph and drag it to the right to expose the data that lies behind it.

Sample Selection

You can select the samples to be displayed, as follows:

- To graph an individual sample, highlight it by clicking on the sample name.
- To display multiple samples, use shift-click.
- To selectively add or remove displayed samples, use control-click.

Data Retrieval

Any data displayed on the Melting Peaks Screen can be retrieved as either a text report, a graphic, or in spreadsheet format:

- From the **Report** pull-down menu, choose the"Summary Report" option. This will generate a text report listing all the run parameters, all the analysis parameters, and all sample names and concentrations.
- To obtain a hard copy of one of the displayed graphs, select one of the following from the **Report** menu: the "Melting Curve" graph from **Step 1**, the"Melting Peak" graph from **Step 1**, the "Peak Area" curve from **Step 2**, or the "Manual Tm" graph from **Step 3**.
- To obtain the data in a tab-delimited spreadsheet format select"Export" from the **Melting Curves** menu. This will create a text file that can be opened in any spreadsheet program.

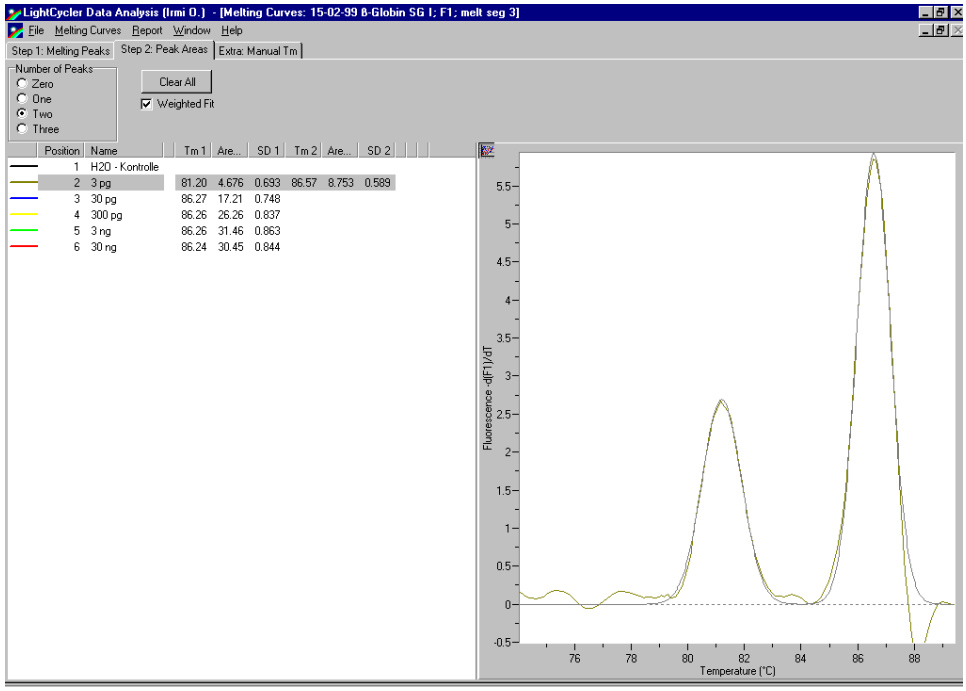
6.3. Melting Curve Step 2: Peak Areas

Introduction

In a well-controlled experiment, peak area can be used to measure relative concentrations. The peak area of an unknown sample can be compared against the peak area of a known standard amplified in the same tube. This method can be used with both hybridization probes and SYBR Green I. It may also be used for relative contribution of mutated vs. Wildtype sequences to signal levels in Mutation Detection experiments.
Caution: In order to compare peak areas between samples, you must minimize non-specific fluorescence. If you selected a calculation method with background subtraction in the previous screen, you may reduce background fluorescence levels in this screen to baseline level. This will allow you to make a valid comparison of melting peak areas.

Peak Area Analysis

The Melting Peak Analysis screen displays melting curve data, which is carried over from the previous screen. In this screen, you may perform a best fit analysis of up to three peaks per sample. Because the number of peaks in each sample may be different, you must analyze each sample individually.


LightCycler Data Analysis (Irmi O.) - [Melting Curves: 15-02-99 B-Globin SG I; F1; melt seg 3]
File Melting Curves Report Window Help
Step 1: Melting Peaks
Step 2: Peak Areas
Extra: Manual Tm
Number of Peaks
Zero
One
Two
Three
Clear All
Weighted Fit
Position Name Tm 1 Are... SD 1 Tm 2 Are... SD 2
1 H20 - Kontrolle
2 3 pg 81.20 4.676 0.693 86.57 8.753 0.589
3 30 pg 86.27 17.21 0.748
4 300 pg 86.26 26.26 0.837
5 3 ng 86.26 31.46 0.863
6 30 ng 86.24 30.45 0.844
Fluorescence -d(F1)/dT
Temperature (°C)

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6.3. Melting Curve Step 2: Peak Areas, Continued

Sample Display

After you select each sample, you will use the box above the graph to define the **Number of Peaks** to be fitted to the data for that sample.
Data for peak areas will be displayed in columns of the sample table (to the left of the graph). The sample table has the following headings: **sample number, name, area, melting temperature** (of each peak), and **standard deviation** (of each peak).

Tip: If you cannot see all the columns of the sample table, click on the left-hand boundary of the graph and drag it to the right to uncover the data that lies behind it.

Change Graph Settings

To change any axis values of the graphs displayed click on the button on the top left corner of the graph.
This will bring up a **Customize Graph** screen. In this screen the user can define values for the axis which will then be carried into the LCDA display. Deselect the **Automatic Fit** option and enter values for the axis in the appropriate fields.

Data Retrieval

Any data displayed on the Peak Areas Screen can be retrieved as either a text report, a graphic, or in spreadsheet format:

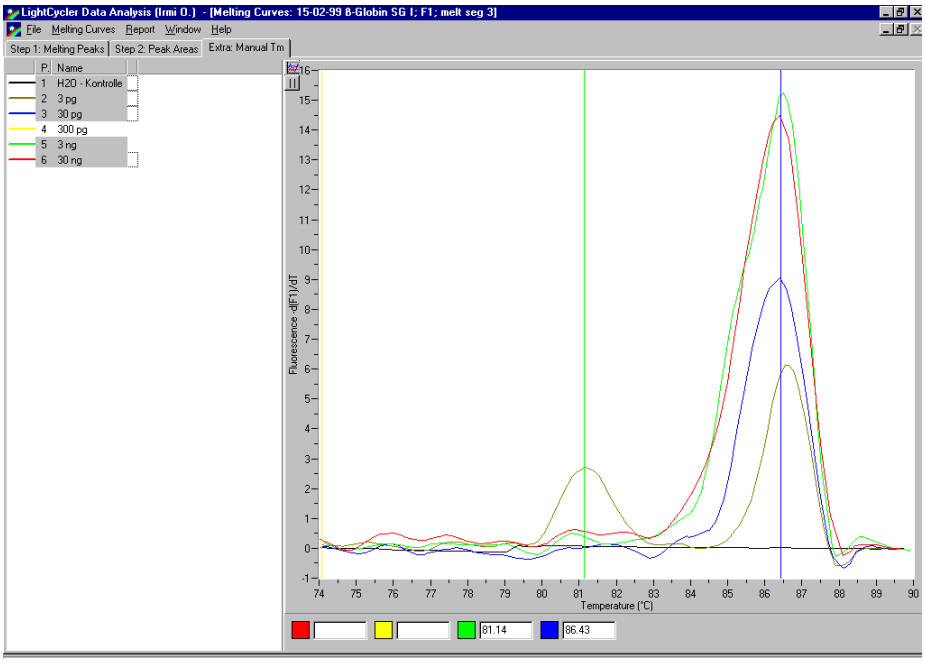
- From the **Report** pull-down menu, choose the"Summary Report" option. This will generate a text report listing all the run parameters, all the analysis parameters, and all sample names and concentrations.
- To obtain a hard copy of one of the displayed graphs, select one of the following from the **Report** menu: the "Melting Curve" graph from **Step 1**, the"Melting Peak" graph from **Step 1**, the "Peak Area" curve from **Step 2**, or the "Manual Tm" graph from **Step 3**.
- To obtain the data in a tab-delimited spreadsheet format select "Export" from the **Melting Curves** menu. This will create a text file that can be opened in any spreadsheet program.

Continued on next page

6.4. Melting Curve Step 3: Manual Tm

Introduction

This screen contains a graph of the melting peaks (carried over from **Step 1**). In this screen, you may position up to four color-coded cursors on the melting peaks.
As each cursor is set on a sample melting peak, the software calculates the melting temperature (i.e., the top of the melting peak) for the sample. The numerical value for the melting temperature will be displayed below the graph (in the box next to the cursor color).



Setting the Cursors

Cursors can also be set to precise positions by using the option at the top left of the graph.
Double click on the temperature displayed next to the cursor color and enter the desired value. The cursor will automatically move to the desired set point.

Change Graph Settings

To change any axis values of the graphs displayed click on the button on the top left corner of the graph.
This will bring up a **Customize Graph** screen. In this screen the user can define values for the axis which will then be carried into the LCDA display. Deselect the **Automatic Fit** option and enter values for the axis in the appropriate fields.

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6.4. Melting Curve Step 3: Manual Tm, Continued

Data Retrieval

Any data displayed on the Extra: Manual Tm Screen can be retrieved as a graphic report:

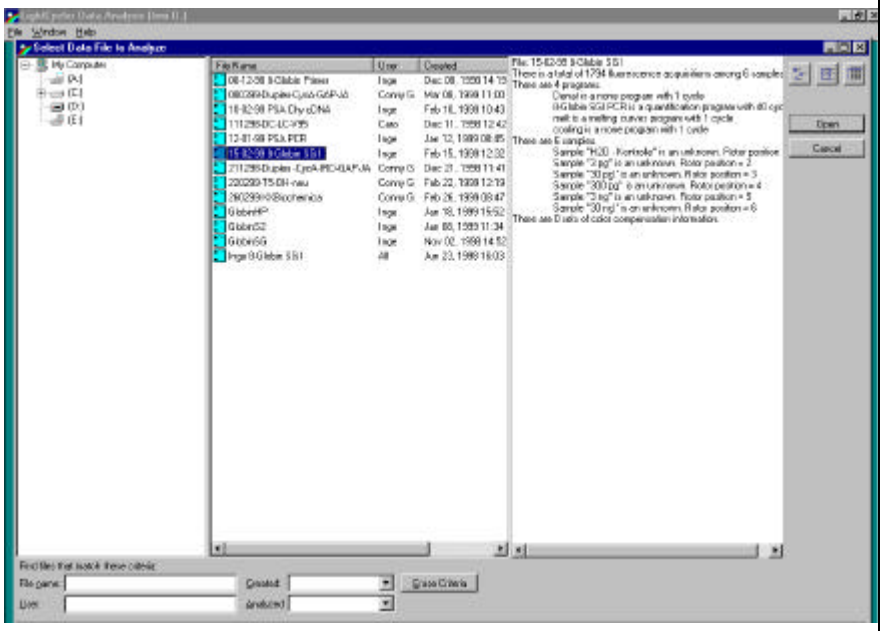
- From the **Report** pull-down menu, choose the"Summary Report" option. This will generate a text report listing all the run parameters, all the analysis parameters, and all sample names and concentrations.
- To obtain a hard copy of one of the displayed graphs, select one of the following from the **Report** menu: the "Melting Curve" graph from **Step 1**, the"Melting Peak" graph from **Step 1**, the "Peak Area" curve from **Step 2**, or the "Manual Tm" graph from **Step 3**.
- To obtain the data in a tab-delimited spreadsheet format select "Export" from the **Melting Curves** menu. This will create a text file that can be opened in any spreadsheet program.

6.5. A Procedure for Analyzing Melting Curve Data

Melting Curve Analysis

Use the procedure below to set up and perform a melting curve analysis on fluorescence data.

Note: Data for this analysis should have been acquired (measured) continuously to generate a high-resolution melting curve.

Step	Action
1	• Click the **Data Analysis** button on the **LightCycler Front Screen** (or choose "LCDA" from the **Analysis** menu). • Open the **Data** file to be analyzed. ***Remember:*** Every user has an individual data folder. You can see only data files belonging to your user name. ***Result:*** The data file you select will open and be displayed on the **LCDA Front Screen.** 

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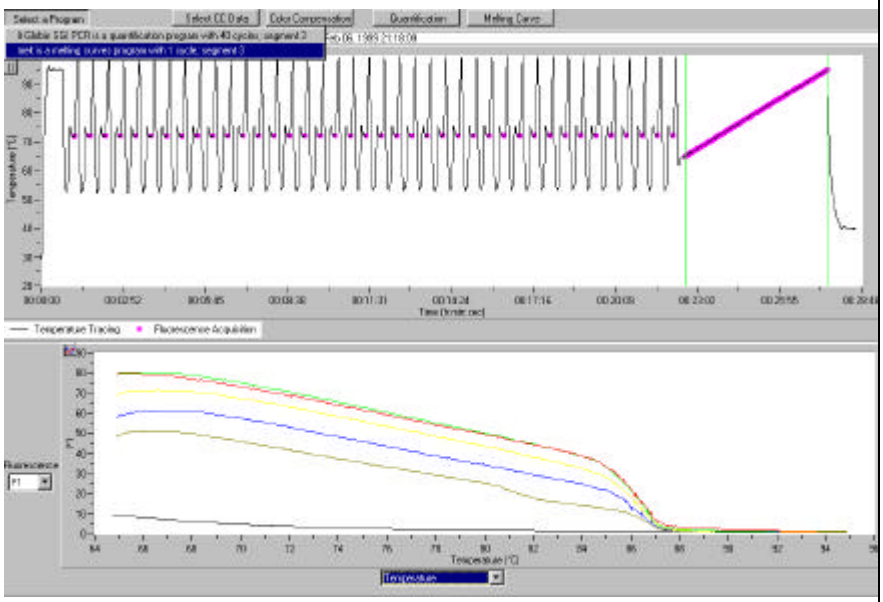
6.5. A Procedure for Analyzing Melting Curve Data, Continued

Melting Curve Analysis (continued)

Step	Action
2	In the upper **Temperature vs. Time** graph, define the melting curve segment of the Experimental Protocol. You may do this in either of two ways: • Define the segment automatically, as follows. • Click on the **Select a Program** button at the top left of the screen. • Choose "Melting Curve Program" to automatically make the cursors bracket the melting curve segment shown on the upper graph. ***Result:*** The x-axis in the lower graph switches automatically to **Temperature.** • Define the segment manually, as follows: Drag the green cursors until they bracket the melting curve segment shown on the upper graph. Select **Temperature** as the x-axis scale for the lower graph (in the pull-down menu below the graph). 

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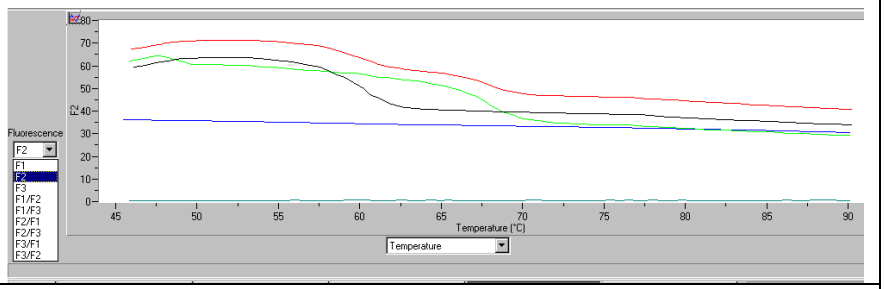
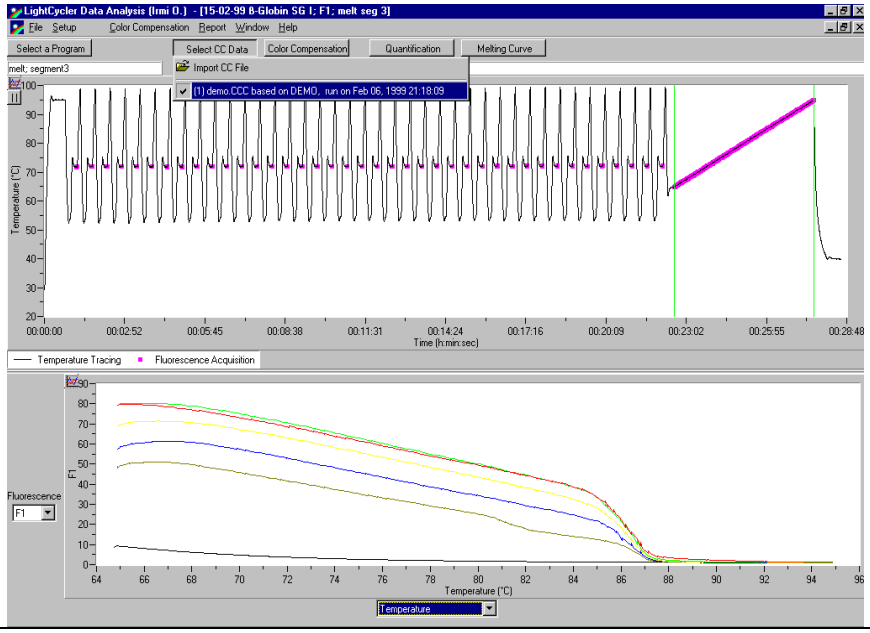
Melting Curve Analysis (continued)

Step	Action
3	In the pull-down menu to the left of the lower graph, select the fluorescence display method appropriate for your data. ***Examples:*** Choose F1 for SYBR Green I, F2 for hybridization probes with an LC-Red 640 label, and F3 for hybridization probes with an LC-Red 705 label. 
4	• If color compensation is required, click on the **Select CC Data** button (at top of screen) and click on the **ccc data file** to be used. ***Note:*** You can also choose "Load Calibration" under the **Color Compensation** pull-down menu and select the **ccc** file to be used. • Click on the **Color Compensation** button (next to the **Select CC Data** button) to activate the color compensation software. 

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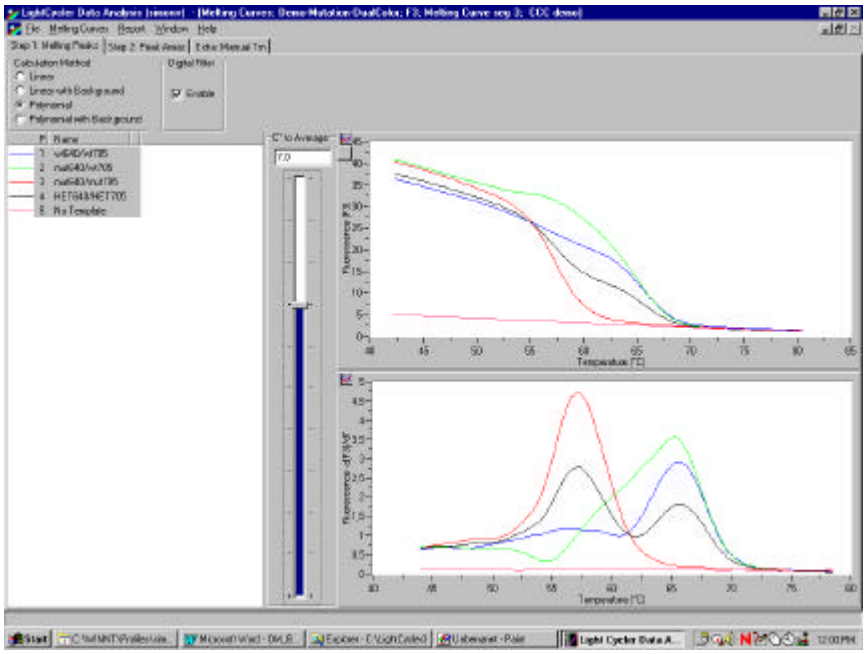
6.5. A Procedure for Analyzing Melting Curve Data, Continued

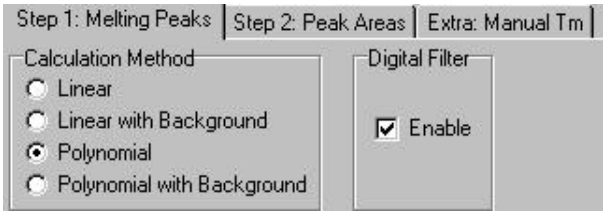
Melting Curve Analysis (continued)

Step	Action
5	Click on the **Melting Curve** button at the top of the screen. ***Note:*** You may also choose "Melting Curve Analysis" in the **Setup** menu. ***Result:*** The melting curve analysis software will be activated and the **Step 1: Melting Peaks Screen** will appear. This screen displays two graphs. The upper graph shows melting curve data as **Fluorescence vs. Temperature.** The calculated **negative derivative plot** of the same data is shown on the lower graph. ***Note:*** The data in the lower graph displays "melting peaks" for each of the amplified products. 
6	In the sample table to the left of the graphs, highlight all the samples to be analyzed.

Continued on next page

Melting Curve Analysis (continued)

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>7</td><td>Select a Calculation Method from the four options in the box at the top left corner of the screen:

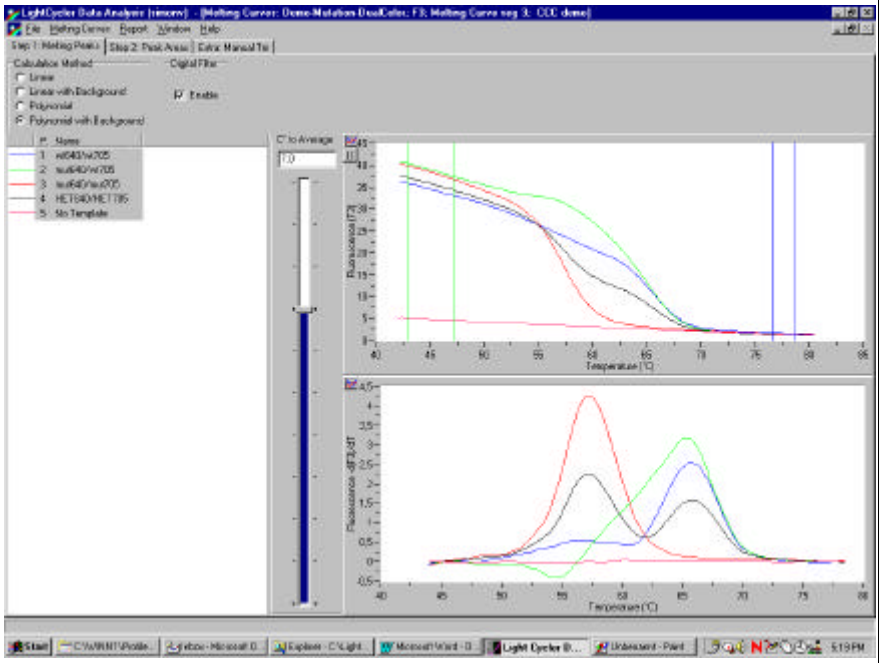
Guidelines for choosing the appropriate option are given in the table below.
<table>
<tr><th>Calculation method</th><th>Description</th></tr>
<tr><td>Linear</td><td>This method just serves for backward compatibility. It correspond to melting curve calculation method in the software version 1.2. This method does not produce a derivative estimate as accurate as the polynomial fit.</td></tr>
<tr><td>Polynomial
(default option)</td><td>This calculation method is the more robust method than the linear fit and thus recommended for all cases of Melting Curve Analysis.</td></tr>
<tr><td>Linear with Background
or
Polynomial with Background</td><td>With Background is an “enhancement” of the first two methods. Background subtraction compensates for the non-specific effects of temperature on fluorescence.

<u>Note:</u> Background subtraction is especially important for the calculation peak areas during Melting Curve analysis.</td></tr>
</table>
<u>Note:</u> For most data, we recommend choosing Polynomial with Background.</td></tr>
</table>

Continued on next page

6.5. A Procedure for Analyzing Melting Curve Data, Continued

Melting Curve Analysis (continued)

Step	Action
8	If you select a method with background subtraction in Step 7, four vertical cursors will appear on the **Temperature vs. Fluorescence** graph: • **End Cursors (blue):** Move these cursors into the flat floorescence region that occured after all DNA melted. These cursors determine the final fluorescence background. ***Note:*** The distance between the cursors is not important. • **Beginning Cursors (green):** Move these cursors into the fluorescence region that was measured before any product melted. These cursors determine the pure effect of temperature on the fluorescent signal. ***Note:*** The distance between the cursors should be as wide as possible without including any melt data. ***Caution:*** Do not include rising regions of the curve in which annealing was still taking place. 

Continued on next page

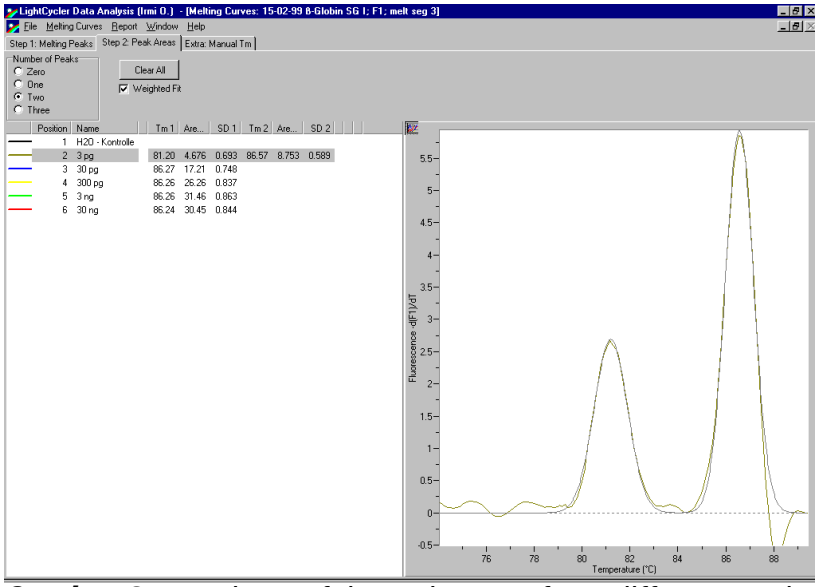
6.5. A Procedure for Analyzing Melting Curve Data, Continued

Melting Curve Analysis (continued)

Step	Action
9	To obtain smoother data curves, click on the "Enable" box to enable the **Digital Filter**. ***Recommendation:*** Almost always enable this filter. ***Exception:*** Do not enable the filter when analyzing melting curves with a lot more data points (e.g. few samples monitored continuously) or a lot fewer data points (e.g. data acquired only once per °C) than a typical melting curve (e.g., melting curve analysis of a full rotor of samples, with 0.1°C stepwise acquisition).
10	Click the **Step 2: Peak Areas** tab at the top of the screen**.** ***Result:*** The **Peak Areas Screen** appears. This screen displays the same melting peak graph that appeared on the previous screen. In this screen, you will be able to calculate peak area and melting temperatures for each sample.

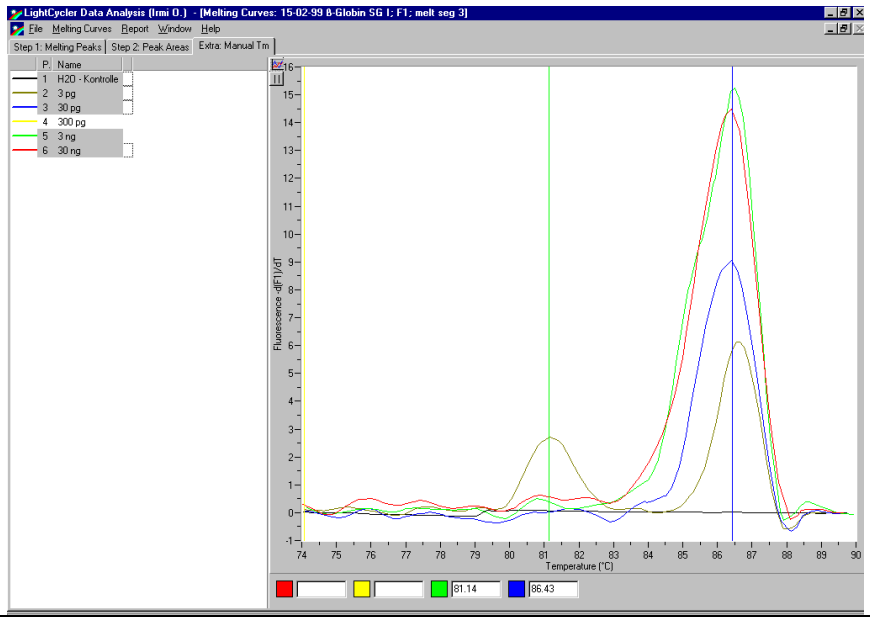
Continued on next page

Melting Curve Analysis (continued)

Step	Action
11	Because the number of melting peaks in each sample may be different, set up the analysis for each sample separately: • In the sample table, highlight one sample at a time, and • In the **Number of Peaks** box (upper left corner of screen), click on the number of peaks in that sample to be subjected to a "best fit" analysis. ***Note:*** You may analyze up to three peaks per sample. ***Result:*** The sample table at the left of the graph will list, for each sample, the results of the analysis. The displayed data include the calculated melting temperatures ($\mathbf{T_m}$s) of all amplification products in the sample, the integrated areas under each melting peak (**Area**), and the standard deviation (**SD**) of each area calculation. ***Tip:*** If all the table columns described are not visible, click on the left-hand boundary of the graph and drag it to the right to expose the data that lies behind it.  ***Caution:*** Comparisons of the peak areas of two different products (e.g. as a measure of relative concentration) will not be valid unless the amplification efficiency of both products is approximately equivalent. This should be confirmed experimentally, if possible.

Continued on next page

Melting Curve Analysis (continued)

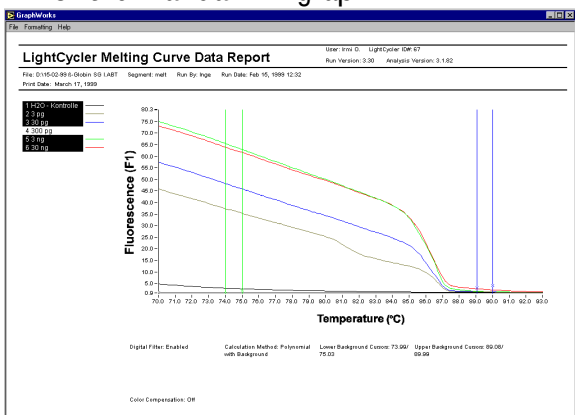
Step	Action
12	• Click the **Extra: Manual Tm** tab at the top of the screen. ***Result:*** The **Manual Tm Screen** appears. • To determine the melting temperature of an amplified product, move one of the four color-coded cursors over the corresponding melting peak. ***Result:*** The numerical value for the T_m will be displayed below the graph (in the box next to the cursor color). This value represents the top of the melting peak. ***Tip:*** This window displays data from all the samples simultaneously. You may set up to a maximum of four cursors on the peaks in this window. 

Continued on next page

6.5. A Procedure for Analyzing Melting Curve Data, Continued

Melting Curve Analysis (continued)

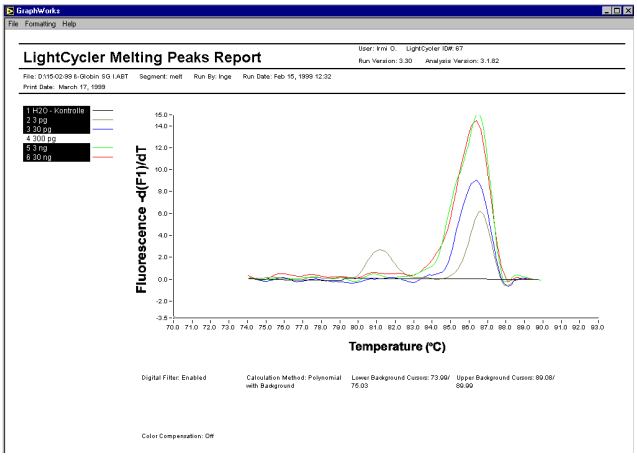
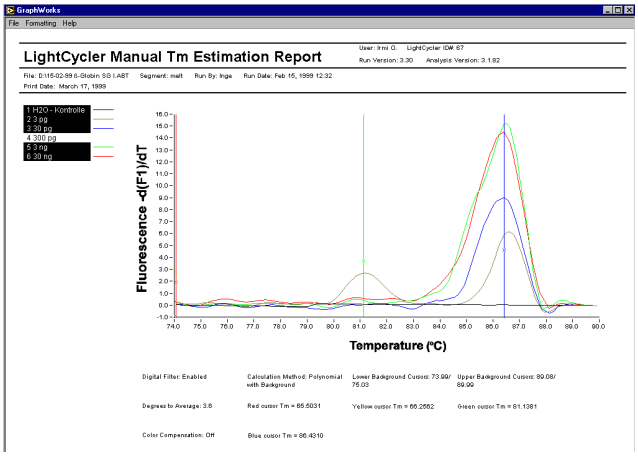
Step	Action

13	• From the **Report** pull-down menu, choose the **"**Summary Report" option. This will generate a text report (shown below) listing all run parameters, analysis parameters, sample names and melting temperatures, and cursor settings:
	  • For a hard copy of the data, select "Print Window" from the pull-down **File** menu at the top of the screen.
	  • For a tab-delimited text file of the data, which may be opened in any spreadsheet program, select "Export" from the **Melting Curves** menu.
	• To obtain a hard copy of one of the graphs (shown below), choose the appropriate graph in the **Report** pull-down menu. Options include the **Melting Curve** graph from Step 1, the **Melting Peak** graph from Step 1, the **Peak Area Curve** from Step 2 or the **Manual Tm** graph.
	 

Continued on next page

6.5. A Procedure for Analyzing Melting Curve Data, Continued

Melting Curve Analysis (continued)

Step	Action
13 (cont.)	 
14	To end the Melting Curve analysis, select “Close this Window” from the pull-down **Window** menu at the top of the screen.

Chapter C
Working with the LightCycler Instrument

1. Working with the LightCycler Instrument

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1.2 Introduction

1.2.1 Principle of LightCycler Technology

System Description

The LightCycler instrument basically consists of two different components: a cycler component (see Section 1.2.2.1) and a fluorimeter component (see Section 1.2.2.2). These components work together to simplify such complex applications as product analysis, quantification, and mutation analysis.
The cycler component has been optimized for rapid PCR applications. Unlike conventional PCR, in which cycling programs take several hours, PCR analyses conducted with the LightCycler take only 20–30 min.

Flexibility of Detection

During PCR, the optimized thermocycler–fluorimeter combination not only permits data to be displayed and analyzed online, but also provides great flexibility to users. For example, by choosing different fluorescent dyes and detection probes (see Section 1.3), the user can perform either:

- Sequence-independent fluorescent detection with double-stranded DNA binding dye SYBR Green I (see Section 1.3.1), or
- Sequence-specific fluorescent detection with oligonucleotide hybridization probes that are coupled to suitable fluorophores (see Section 1.3.2).

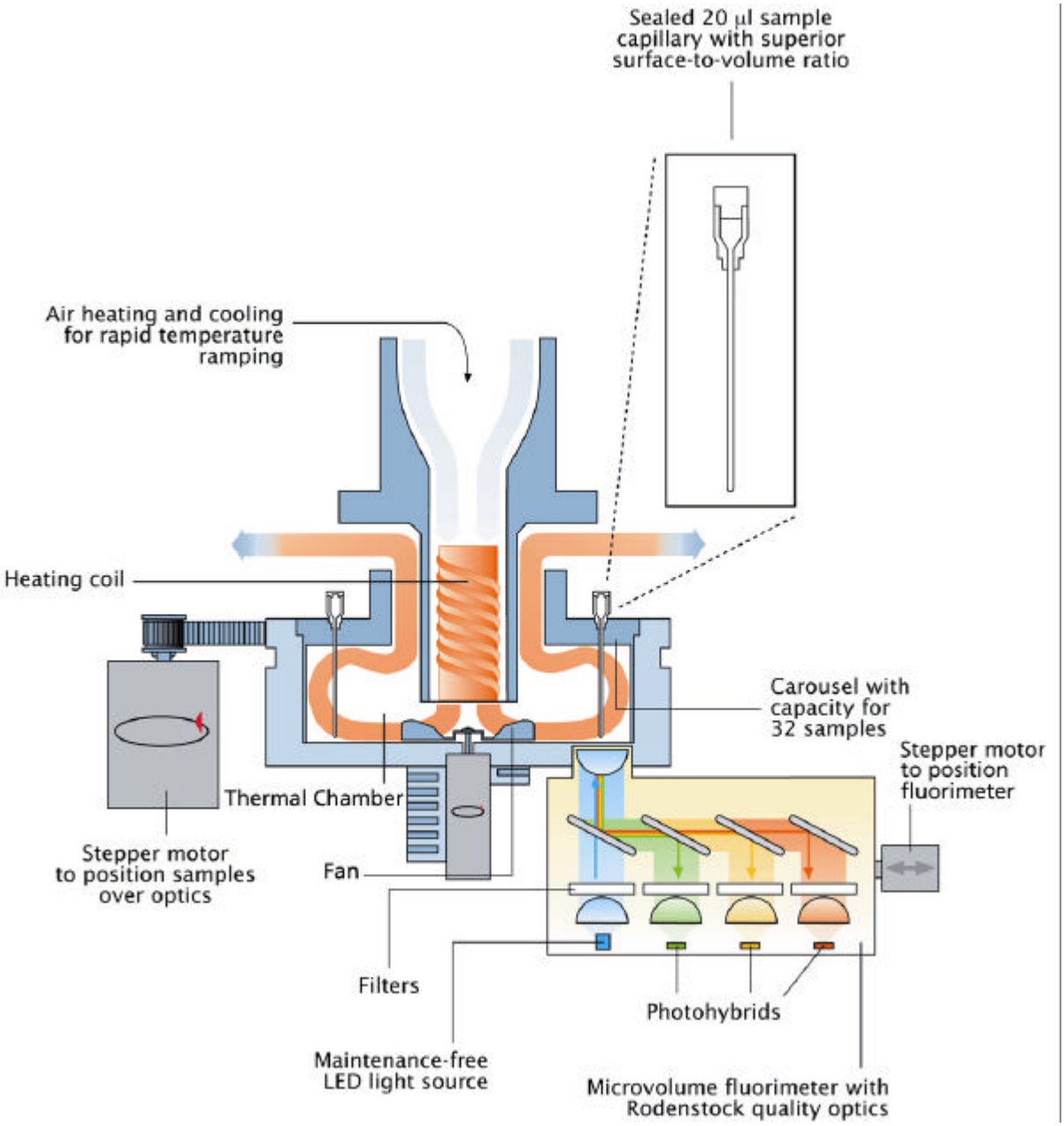
Analysis of Results

Data obtained from fluorimetric analysis can be displayed and directly evaluated with the special LightCycler software (see chapters B and D). Hence, no additional tedious and time-consuming procedures (e.g., agarose gel or Southern blotting analyses) are required to analyze the PCR product.
Direct, uncomplicated analysis of results also reduces the number of sample handling steps, thus minimizing the risk of sample contamination.

1.2.2 Instrument Components of the LightCycler

1.2.2.1 The Cycler Component

Schematic of the LightCycler


Sealed 20 µl sample capillary with superior surface-to-volume ratio
Air heating and cooling for rapid temperature ramping
Heating coil
Carousel with capacity for 32 samples
Stepper motor to position fluorimeter
Thermal Chamber
Stepper motor to position samples over optics
Fan
Filters
Photohybrids
Maintenance-free LED light source
Microvolume fluorimeter with Rodenstock quality optics

Continued on next page

1.2.2.1 The Cycler Component, Continued

Sample Carousel

The sample carousel is the central element within the cylindrical thermal chamber. The carousel holds 32 samples in glass capillaries (outer diameter, 1.55 mm; length, 35 mm). These glass capillaries also serve as cuvettes for fluorimetric determination of the PCR products formed.
Note: High-speed cycling in the LightCycler is made possible, in part, by the design of these capillaries. These specially manufactured glass capillaries permit reaction volume to be reduced to 10–20 µl. Their optimized surface-to-volume ratio guarantees extremely rapid thermal transfer within the reaction mixture. As a result, the temperature transition ("ramping") times (e.g., the time required for the transition from 94°C to 55°C after sample denaturation) during PCR are very fast and the total time required for each cycle is about 15–20 seconds.

Thermal Chamber

The thermal chamber is directly connected to the optical system of the fluorimeter. Temperatures within the chamber are controlled by an integrated measuring system equipped with a temperature sensor, which is installed near the sample capillaries in the carousel.
A blower, equipped with separate heating coils, supplies the cylindrical thermal chamber with hot air and ambient temperature air. A high-velocity fan, located at the base of the thermal chamber, evenly distributes the incoming air. This circulating air creates an even temperature throughout the thermal chamber. Surplus air is vented through an opening at the side of the chamber.
Note: The use of air as a heat-transfer medium also contributes to the high-speed cycling capabilities of the LightCycler.

1.2.2.2 The Fluorimeter Component

Optics

The optical fluorescence components of the LightCycler are schematically illustrated on page C4. These include:

- A blue, high-performance diode (blue LED) serves as the energy source for sample excitation. Light emitted by the diode is filtered and collimated (brought to the same wavelength) by a special optical system to produce 1 mW of light at a wavelength of 470 nm. The homogeneous, 470 nm light beam is subsequently focused onto the individual glass capillaries and hence onto the samples to be measured in the sample carousel.
- A high precision stepper motor rotates the sample carousel to bring the glass capillaries into the optimum position for excitation and measurement.
- Dichroic mirrors divert the light emitted from the sample into one of three detection channels.
  Note: The channel that receives the light depends on the spectral properties of the fluorophore used.
- A special optical filtering system within each of these channels provides additional collimating and spectral concentration of the emitted light beam, eventually focusing the light beam onto a photohybrid for final evaluation.

Detection Channels

The three detection channels within the LightCycler fluorimeter are equipped with combination filters. These permit analyses at certain emission wavelengths, which allow exact measurement of emissions from the fluorophores shown in the table below:

Fluorophore	Excitation Channel	Detection Channel		
		1	2	3
	470 ± 20 nm	530 ± 10 nm	640 ± 10 nm	710 ± 20 nm
Fluorescein	493 nm	525 nm		
SYBR Green I	497 nm	521 nm		
LC-Red 640*	–		640 nm	
LC-Red 705*	–			705 nm

*LC-Red 640 = LightCycler-Red-640-N-hydroxy-succinimide ester (Cat. No. 2 015 161); LC-Red 705 = LightCycler-Red-705-Phosphoramidite (Cat. No. 2 157 594). LC-Red 640 and LC-Red 705 are not excited by the blue LED, but are FRET partners of fluorescein as described in Section 1.3.2.

1.2.3 LightCycler Accessories

Introduction

In this section, you will learn more about the accessories that are supplied with the LightCycler.

Tailor-made Accessories

You will receive the following accessories with the LightCycler:

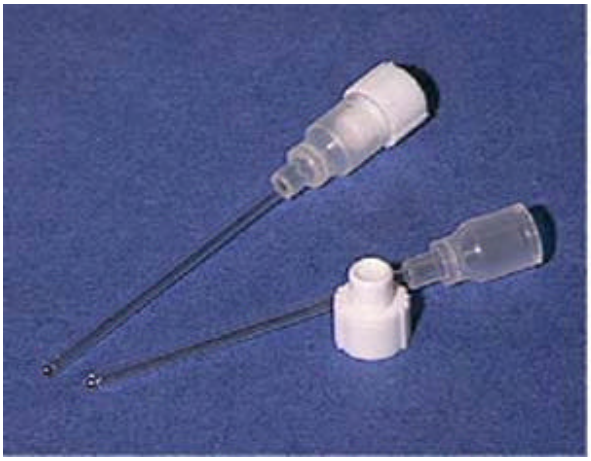
- 1 cooling block,
- 32 adapters which hold the capillaries during pipetting and centrifugation,
- 1 box containing 96 sample capillaries.

Accessory	Description
	The **cooling block** provides 32 positions for adapters, 6 positions for 1.5 ml reaction tubes, and 6 positions for 0.5 ml reaction tubes. It keeps both your reagents, the capillaries, and the adapters cool during pipetting. When the cooling block is pre-cooled to 4°C, it will maintain this temperature for 30 min while sitting at room temperature. After the cooling block sits an additional 30 minutes at room temperature, its temperature will be approx. 10°C. ***Note:*** Do not pre-cool the block in a freezer (–20°C) because your samples will immediately freeze when you put them into the block.
	The **adapters** hold the capillaries during pipetting and centrifugation.

Continued on next page

1.2.3 LightCycler Accessories, Continued

Tailor-made Accessories (continued)

Accessory	Description
	**Sample capillaries** permit ultrarapid cycling because they have a high surface-to-volume ratio. Each capillary is about 35 mm long and has an outer diameter of 1.55 mm; the optimal sample volume is 10–20 µl. A single capillary consists of three parts: • The glass capillary itself, • A plastic "reservoir" at the top of the capillary, and • A plastic stopper to seal the capillary. ***Note:*** Samples are pipetted into the capillary reservoir and then forced into the glass capillary tube by centrifugation.

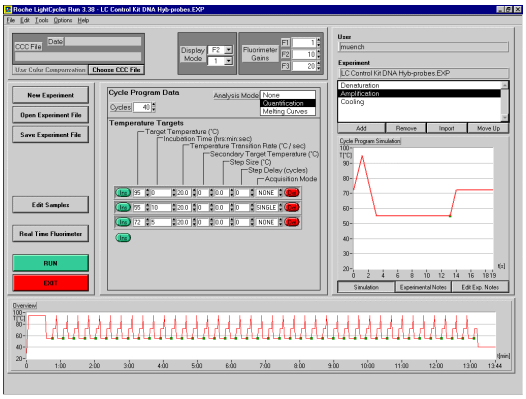
1.2.4 Handling the Capillaries

Introduction

This section will provide an overview of how to prepare capillaries for a LightCycler run and how to remove broken capillaries.

Filling the Capillaries

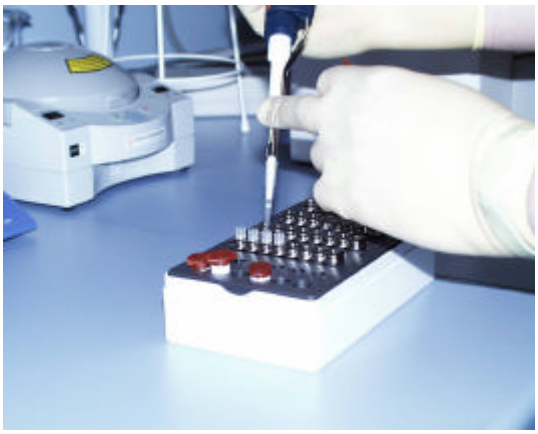
The following table gives you a brief description of how samples are prepared and loaded into the capillaries.

Step	Action	
	Picture	Description
1		Using the LightCycler software, program the experimental protocol and define the sample numbers, names etc. (see Chapter B).
2		Prepare a master mix containing all the reaction reagents except the DNA/RNA template (see Chapter D). Pipette all reagents into a 1.5 ml or 0.5 ml reaction tube and mix by gentle vortexing.

Continued on next page

1.2.4 Handling the Capillaries, Continued

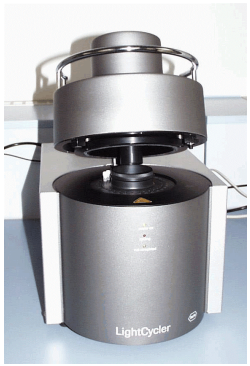
Filling the Capillaries (continued)

Step	Action	
	Picture	**Description**
3		Place capillaries into adapters that have been pre-cooled in the cooling block. Pipette the reagent mix into the plastic container at the top of the capillary. Add the DNA/RNA template to the capillary. Seal the capillary with a plastic stopper.
4		To transfer the reaction mix from the plastic container at the top of the capillary into the glass tube, centrifuge the adapters (which contain the capillaries) briefly in a standard benchtop centrifuge at 700 x g. ***Note:*** Use only rotors that are designed to hold 2.0 ml reaction tubes. Do not exceed a centrifugation force of 700 x g.
5		Remove the glass capillaries from the adapters and place them into the LightCycler sample carousel.

Continued on next page

1.2.4 Handling the Capillaries, Continued

Filling the Capillaries (continued)

Step	Action	
	Picture	**Description**
6		Place the carousel containing the samples in the LightCycler and close the lid. ***Note:*** You are now ready to start the run.

Continued on next page

1.2.4 Handling the Capillaries, Continued

Effects of Broken Capillaries

If a capillary breaks or cracks while in the sample carousel, you must remove the broken glass and clean the sample hole thoroughly. If a new capillary is inserted into that hole before it is cleaned, residual glass fragments can damage the precision bore of the hole. Such damage may alter the proper optical alignment of that sample hole and affect the accuracy of future data.

How to Remove Broken Capillaries

Follow the cleaning instructions below to remove a broken sample capillary.

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Remove the sample carousel from the LightCycler.</td></tr>
<tr><td>2</td><td>Starting from either the top or the bottom of the carousel, carefully remove capillary fragments from the sample carousel hole.</td></tr>
<tr><td>3</td><td>Depending on the amount of contamination, remove fine glass particles as follows:
<table>
<tr><th>IF the sample carousel...</th><th>THEN...</th></tr>
<tr><td><u>IS NOT</u> contaminated with liquid</td><td>• Carefully remove all glass splinters.
• Clean the sample hole with the microbrush (provided with the LightCycler).</td></tr>
<tr><td><u>IS</u> contaminated with liquid</td><td>• Flush hole with running water to remove glass splinters.
• While rinsing the hole with water, clean the sample hole with the microbrush (provided with the LightCycler).
• Allow the sample carousel to dry thoroughly before reusing.
• Optional: Treat the sample carousel for nucleases (e.g., with DEPC, or Velcorin) and then autoclave.</td></tr>
</table>
</td></tr>
</table>

Note: In place of the microbrushes provided with the LightCycler, you may use interdental brushes (e.g., Oral B interdental brushes, available in drugstores or pharmacies).

1.3 Procedures for Fluorimetric Online Detection

Introduction

There are two different methods for online detection and evaluation of fluorimetric PCR reactions in glass capillaries. The PCR products formed may be detected via either:

- Fluorophores that bind to all double-stranded DNA molecules regardless of sequence, e.g., the double-stranded DNA binding dye SYBR Green I (see Section 1.3.1), or
- Fluorophores, e.g., LightCycler-Red 640 and Fluorescein, coupled to sequence-specific oligonucleotide hybridization probes that only detect certain PCR products (see Section 1.3.2).

1.3.1 Sequence-Independent Detection with SYBR Green I

Method

SYBR Green I is a dye that binds specifically to double-stranded DNA. Its inherent fluorescence is enhanced when it binds to the minor groove of double-stranded DNA.

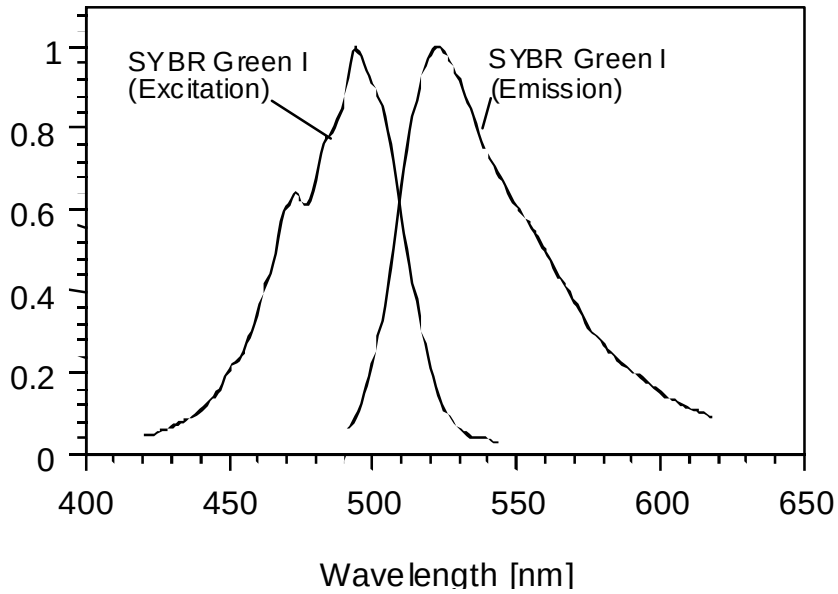
Note: SYBR Green I is a registered trademark of Molecular Probes Inc. (Eugene, OR, USA).

During PCR, SYBR Green I binds to DNA products as they are synthesized. Thus, the increase in SYBR Green I fluorescence, when measured at the end of each elongation cycle, indicates the amount of PCR product formed during that cycle.

Note: To increase the specificity and sensitivity of SYBR Green I detection, perform a melting curve analysis on the amplification reaction (see Section 1.4).

Spectral Characteristics

The maximum excitation of SYBR Green I dye occurs at 497 nm. Maximal emission of DNA stained with SYBR Green I occurs at 521 nm.



Sensitivity

Dye staining can detect from approx. 1 to 10^9 copies of a target sequence. Resolution of this amplification-detection method is limited at very low concentrations of template (see "Nonspecific Background" on the next page).

Fluorimetric Measurement

The LightCycler measures the fluorimetric intensity of SYBR Green I at the end of each elongation phase.

Continued on next page

1.3.1 Sequence-Independent Detection with SYBR Green I,
Continued

Temperature Stability	SYBR Green I is stable at temperatures and pHs that occur during rapid thermal cycling.
Toxicity	In the Ames mammalian microsomal reverse mutation assay, SYBR Green I is significantly less mutagenic than ethidium bromide (Ames BN et al., 1973; Maron DM & Ames BN, 1983).
Nonspecific Background	In the negative assay control, the signal obtained from an initial template amount of 1–100 target sequences is barely above the measurable background. If primer-dimers form in a reaction, the fluorescence signal from the primer dimer amplicons may superimpose on that of the desired target. ***Note:*** To increase the specificity and sensitivity of SYBR Green I detection assays, use melting curve analysis to evaluate the amplification reaction (see Section 1.4).

1.3.2 Sequence-Specific Detection with Hybridization Probes

Method

Sequence-specific hybridization probes provide the most specific real-time analysis of amplified target sequences. This method can even detect single copy sequences in complex nucleic acid templates, for example genomic DNA.
Hybridization probes should be used for quantification with appropriate evaluation software (**Quantification** option; see Chapter B).

Hybridization Probes

The method uses two oligonucleotide hybridization probes, which hybridize to adjacent internal sequences. The 5′ oligonucleotide (donor) has a fluorescein label at its 3′ end. The 3′ oligonucleotide (acceptor) has either LightCycler-Red 640 or LightCycler-Red 705 at its 5′ end.

Advantages

In this detection method, the hybridization probes are highly unlikely to bind artificially amplified sequences. Thus, nonspecific sequences and primer-dimers, which may be amplified due to mispriming, will not emit a measurable signal.

Melting Curve Analysis

Combining hybridization probe detection with subsequent melting curve analysis makes possible such applications as rapid genotyping and mutation analysis (see Section 1.4).
Note: A single mismatch between the internal hybridization probe and its target sequence—caused by a single point mutation— can drastically change the melting temperature of the bound probe.

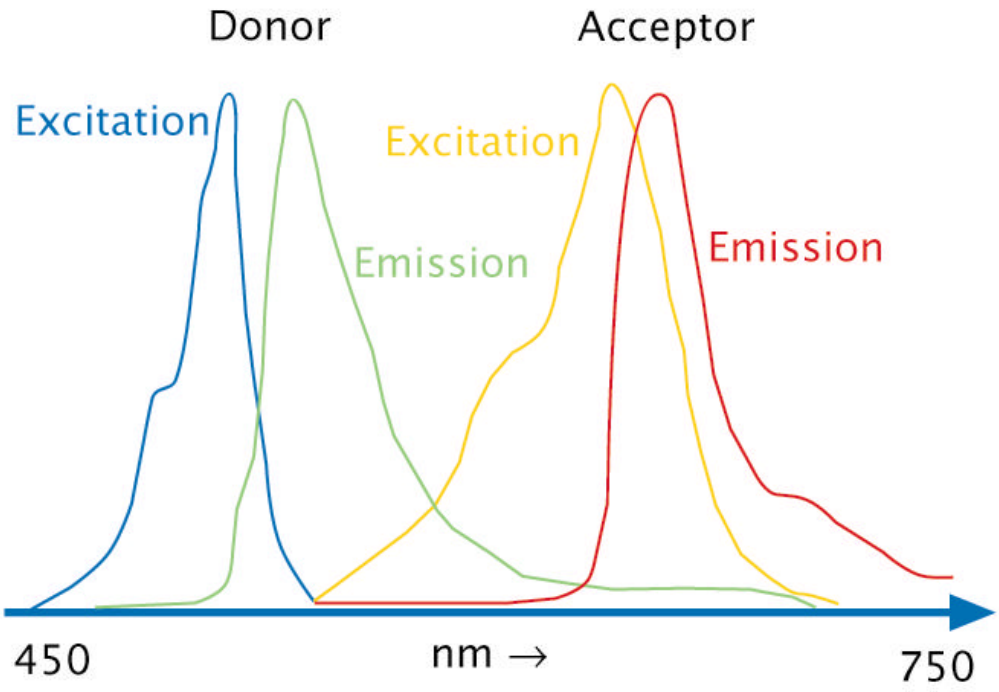
Continued on next page

1.3.2 Sequence-Specific Detection, Continued

Fluorescence Resonance Energy Transfer (FRET)

Fluorescence resonance energy transfer (FRET) is a distance-dependent transfer of energy between two adjacent fluorophores without the emission of a photon. Primary conditions for FRET are:

- Donor and acceptor molecules must be in close proximity (typically 10–100 Å).
- Absorption spectrum of the acceptor must overlap fluorescence emission spectrum of the donor (see figure below).
- Donor and acceptor transition dipole orientations must be approximately parallel.



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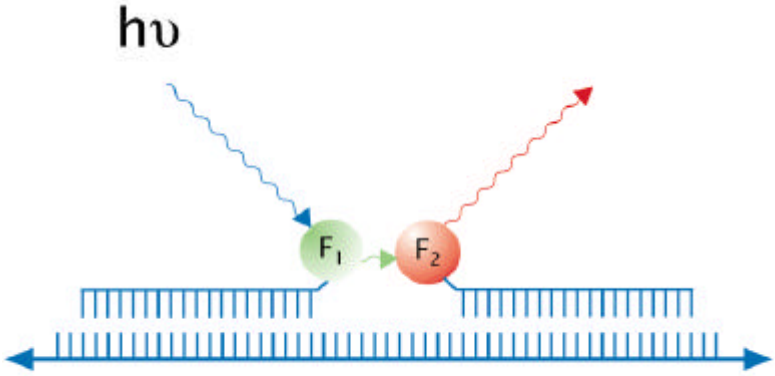
FRET with Hybridization Probes

The following schematic illustrates how two adjacent oligonucleotide hybridization probes produce a measurable fluorescence emission.



During the PCR annealing step, two different oligonucleotides hybridize, head-to-tail, to adjacent regions of the target DNA. The fluorophores, which are directly coupled to the oligonucleotides, are very close in the hybrid structure. The "F_1" donor fluorophore (e.g., fluorescein) is excited by an external light source, then passes on part of its excitation energy to the adjacent "F_2" acceptor (e.g., LightCycler-Red 640) (FRET). The excited F_2 fluorophore then emits measurable light.

1.3.2.1 Hybridization Probe Design

Introduction

The Hybridization Probes method for LightCycler monitoring of DNA amplification requires two adjacent, fluorescently labeled oligonucleotide probes (see Section 1.3.2).
Many of the rules described for primer design (see Section 2.3.2) may be used to design these probe oligonucleotides. However, optimal probe design involves a few substantial differences, which are described below.

Probe Melting Temperature

In general, the T_m of the probes for a given reaction must be 5°–10°C higher than the T_ms of the primers used in that reaction.
Note: There are several methods for calculating the T_m of a hybrid structure. An excellent method is available from the web site of TIB MOLBIOL (www.TIB-MOLBIOL.de/oligo_ag.html).
Caution: Avoid designing extremely stable probes (T_m >10°–20°C higher than primer T_m). Such probes may interfere with the amplification reaction and lower the sensitivity of the assay.

Hybridization Probes for Mutation Detection

If the probes are used to detect mutations, the probe that covers the predicted mutation site must have a lower T_m than the second probe (the anchor probe). This ensures that the fluorescent signal in the assay will be controlled solely by the probe that covers the mutation site.
Note: To generate an anchor probe that has a higher T_m than the "mutation" probe, either make the anchor probe longer than the mutation probe or give the anchor probe a higher G/C content.

Avoid Complementary Probe Sequences

Probes must not hybridize to each other; avoid designing probes that contain complementary sequences.
Note: Do not allow the probes to be complementary to the amplification primers, which could lead to the formation of primer-dimers.

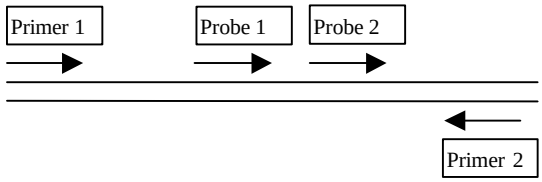
Continued on next page

1.3.2.1 Hybridization Probe Design, Continued

Selection of the Probe Target

In choosing the target sequence to be recognized by the probes, use the following guidelines:

- When annealed to the target sequence, the hybridization probes must sit very close to each other, in a head-to-tail arrangement. For most applications, the gap between the 3′ end of Probe 1 and the 5′ end of Probe 2 should be 1 to 5 bases.
  Recommendation: When designing the probes, start with a gap of one base.
- Choose a target in a "balanced" sequence region. Avoid sequences that:
  - Have a run of Gs and Cs at one end (which tend to hybridize strongly to complementary probes),
  - Are purine (G and A) rich,
  - Are monotonous or repetitive, or
  - Are obviously self-complementary.
- Choose the probe target so it is as far as possible from the 5′ primer-binding site on the same strand. We recommend that the target be close to, but not overlap the primer-binding site on the opposite strand (see diagram below). This placement allows time for fluorescent measurements to be made before the primers are completely extended, and before the probes are displaced by Taq DNA polymerase.



Note: If fluorescence signals are faint after probe hybridization, we recommend changing the primer location as follows:

- Vary the gap length between the probes, or
- Completely relocate both probes to a position near the middle of the amplicon.

Continued on next page

Preventing Probe Extension

Because the probes have a higher melting temperature than the primers, these probes can also serve to prime *de novo* synthesis during amplification. Consequently, to prevent probe extension during PCR, block the 3′ end of both probes as follows:

- Label the 3′ end of **probe 1** with fluorescein (see Section 1.3.2.2); which blocks extension.
- Phosphorylate the 3′ end of **probe 2** to prevent extension. [This probe is labeled at the 5′ end with LightCycler-Red 640 (see Section 1.3.2.3) or LightCycler-Red 705 (see Section 1.3.2.4).]

Labeling

See Sections 1.3.2.2, 1.3.2.3, and 1.3.2.4 for detailed information about labeling probes with the appropriate dyes.

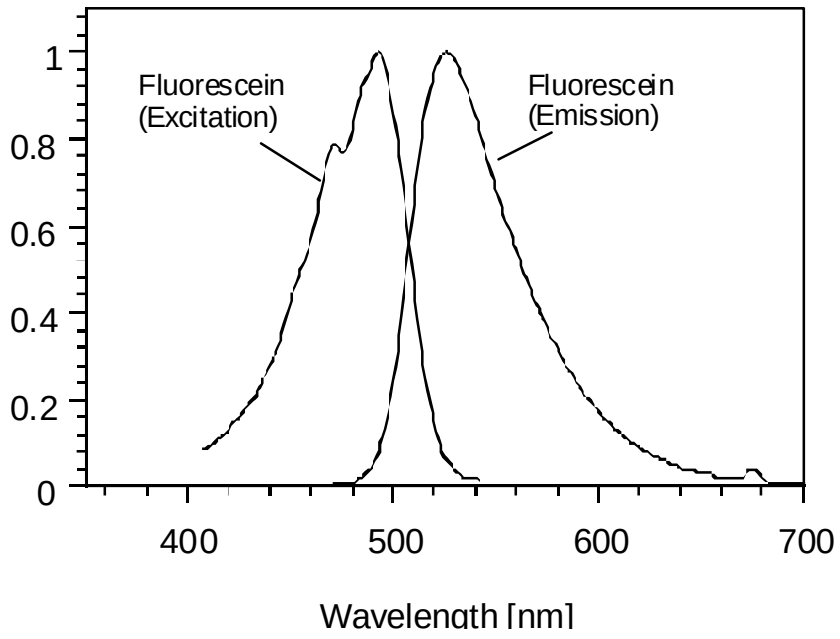
1.3.2.2 Fluorescein: 3′ End Label

Introduction

The upstream ("donor") probe must contain a fluorophore (usually fluorescein) at its 3′ end.

Spectral Characteristics

The maximum excitation of fluorescein occurs at approx. 493 nm; maximum emission of the dye occurs at 525 nm.



Sequence near Label

If possible, do not place the label next to a guanosine residue, since the nucleotide may quench the fluorescence of the label.

Activated Form for Labeling

To label a probe with fluorescein during oligonucleotide synthesis, use an activated form of the fluorophore, e.g. fluorescein-CPG.

Continued on next page

1.3.2.2 Fluorescein: 3′ End Label, Continued

Quality Check

As a check on the quality of the labeled oligonucleotides, we recommend examining their UV/VIS spectrum between 200 and 800 nm. Search for two important peaks:

- A peak at 260 nm, contributed by the oligonucleotide part of the labeled probe, and
- A peak at 493 nm, contributed by the dye moiety.

Based on the UV/VIS absorption spectrum, calculate the ratios of the absorbance values at 493 nm and 260 nm. Depending on the length and sequence of the oligonucleotides, the approximate ratios are:

Length of oligonucleotide	Ratio A_{493}/A_{260}
20-mer	0.25-0.35
25-mer	0.20-0.30
30-mer	0.15-0.25

Custom Synthesis

For custom synthesis of fluorescein labeled oligonucleotides, please contact your local Roche Molecular Biochemicals representative or the following labs:

Country	Source
France	Genset SA 24, rue Royale 75008 Paris, France Phone: +33 1 55 04 59 00 Fax: +33 1 55 04 59 29
Germany	TIB MOLBIOL Syntheselabor Tempelhofer Weg 11-12 D-10829 Berlin, Germany Phone: +49 (030) 787 99455 Fax: +49 (030) 78799499 Email: DNA@TIB-MOLBIOL.de
USA	Genset Corporation 875 Prospect Street, Suite 206 LA JOLLA CA 92037 Phone: +1 619 551 3000 Fax: +1 619 551 3001

1.3.2.3 LightCycler - Red 640: 5′ End Label

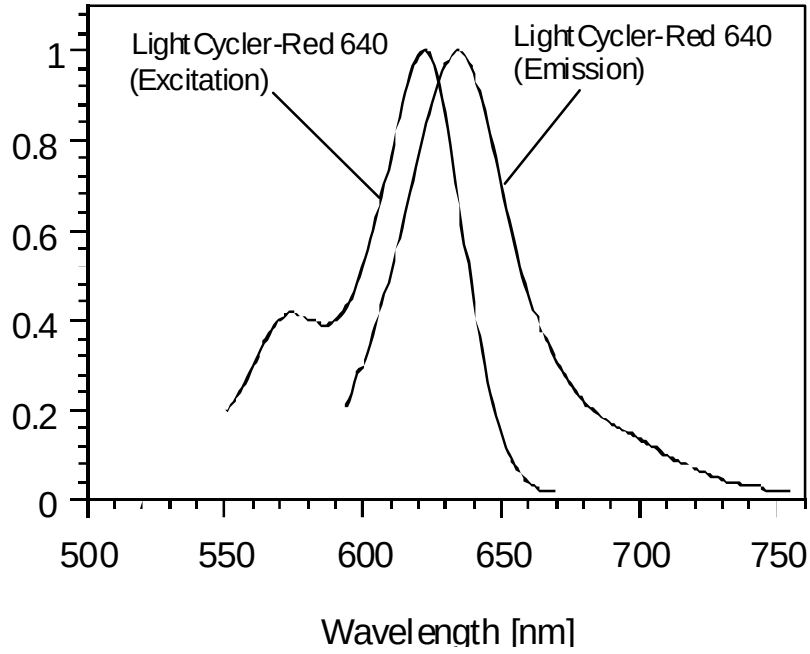
Introduction

The downstream ("acceptor") probe must contain a fluorophore at its 5′ end. One fluorophore commonly used at this site is LightCycler-Red 640 (LC-Red 640). An activated form [N-hydroxysuccinimide (NHS) ester] of LC-Red 640, suitable for labeling oligonucleotides, is offered by Roche Molecular Biochemicals (Cat. No. 2 015 161).

Note: The 3′ end of the acceptor probe must be phosphorylated, to prevent its elongation during PCR (see Section 1.3.2.1).

Spectral Characteristics

The maximum excitation of LC-Red 640 occurs at 625 nm; maximum emission of the dye occurs at 640 nm.



Sequence near Label

If possible, do not place the label next to a guanosine residue, since the nucleotide may quench the fluorescence of the label.

Labeling Principle

5′-amino-substituted -3′-phosphorylated oligonucleotides react with the NHS-ester of LC-Red 640 in a mixture of sodium borate buffer and dimethyl formamide at pH 8.5–9.0.

Note: The labeling procedure is described in detail in the package insert for the NHS ester of LC-Red 640. The pack insert is also available at our Web site (http://biochem.roche.com/pack-insert/2015161.pdf). Custom synthesis laboratories can also synthesize and end-label the required oligonucleotide probes.

Continued on next page

Quality Check

As a check on the quality of the labeled oligonucleotides, we recommend examining their UV/VIS spectrum between 200 and 800 nm. Search for two important peaks:

- A peak at 260 nm, contributed by the oligonucleotide part of the labeled oligonucleotide, and
- A peak at 620 nm, contributed by the dye moiety.

Based on the UV/VIS absorption spectrum, calculate the ratios of the absorbance values at 620 nm and 260 nm. Depending on the length and sequence of the oligonucleotides, the approximate ratios are:

Length of oligonucleotide	Ratio A_{620}/A_{260}
20-mer	0.60-0.65
25-mer	0.43-0.55
30-mer	0.35-0.45

Custom Synthesis

For custom synthesis of LC-Red 640 labeled oligonucleotides, please contact your local Roche Molecular Biochemicals representative or the following labs:

Country	Source
France	Genset SA 24, rue Royale 75008 Paris, France Phone: +33 1 55 04 59 00 Fax: +33 1 55 04 59 29
Germany	TIB MOLBIOL Syntheselabor Tempelhofer Weg 11-12 D-10829 Berlin, Germany Phone: +49 (030) 787 99455 Fax: +49 (030) 787 99499 Email: DNA@TIB-MOLBIOL.de
USA	Genset Corporation 875 Prospect Street, Suite 206 LA JOLLA CA 92037 Phone: +1 619 551 3000 Fax: +1 619 551 3001

1.3.2.4 LightCycler - Red 705: 5′ End Label

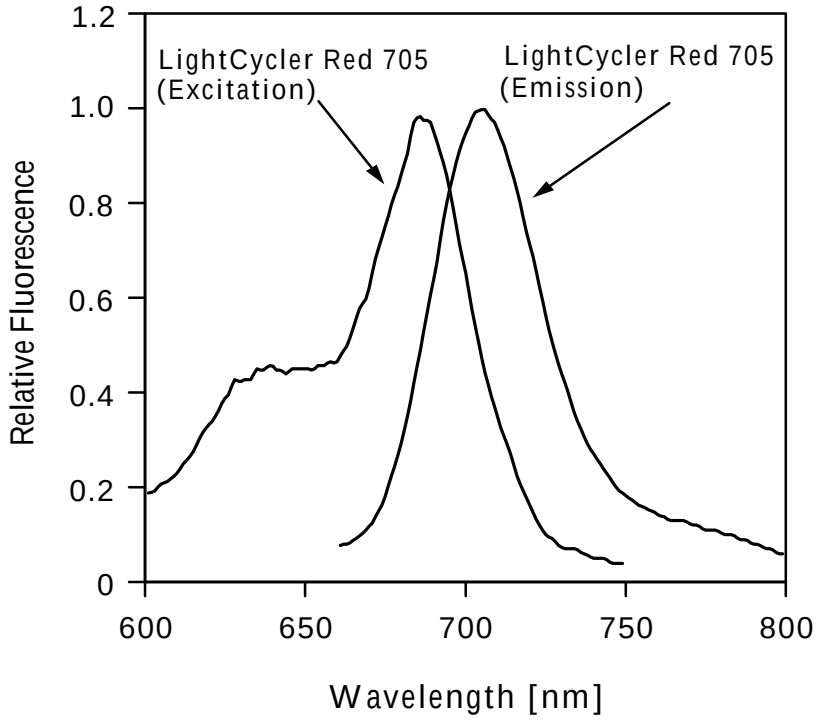
Introduction

The downstream (“acceptor”) probe must contain a fluorophore at its 5′ end. One fluorophore commonly used at this site is LightCycler - Red 705 (LC-Red 705). An activated form (phosphoramidite) of LC-Red 705, suitable for labeling oligonucleotides, is offered by Roche Molecular Biochemicals (Cat. No. 2 157 594).

Note: The 3′ end of the acceptor probe must be phosphorylated, to prevent its elongation during PCR (see Section 1.3.2.1).

Spectral Characteristics

The maximum excitation of LC-Red 705 occurs at 685 nm; maximum emission of the dye occurs at 705 nm.



Sequence near Label

If possible, do not place the label next to a guanosine residue, since the nucleotide may quench the fluorescence of the label.

Labeling Principle

3′- phosphorylated oligonucleotides react with LC–Red 705 – Phosphoramidite during the final synthesis step of solid phase phosphoramidite synthesis.

Note: Labeling procedure is described in detail in the package insert for LC-Red 705-Phosphoramidite. The pack insert is also available at our Web site (http://biochem.roche.com/pack-insert/2157594.pdf). Custom synthesis laboratories can also synthesize and end-label the required oligonucleotide probes.

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Quality Check

As a check on the quality of the labeled oligonucleotides, we recommend examining their UV/VIS spectrum between 200 and 800 nm. Search for two important peaks:

- A peak at 260 nm, contributed by the oligonucleotide part of the labeled oligonucleotide, and
- A peak at 685 nm, contributed by the dye moiety.

Based on the UV/VIS absorption spectrum, calculate the ratios of the absorbance values at 685 nm and 260 nm. Depending on the length and sequence of the oligonucleotides, the approximate ratios are:

Length of oligonucleotide	Ratio A_{685}/A_{260}
20-mer	1.00-1.15
25-mer	0.90-1.00
30-mer	0.75-0.90

Custom Synthesis

For custom synthesis of LC-Red 705 labeled oligonucleotides, please contact your local Roche Molecular Biochemicals representative or the following labs:

Country	Source
France	Genset SA 24, rue Royale 75008 Paris, France Phone: +33 1 55 04 59 00 Fax: +33 1 55 04 59 29
Germany	TIB MOLBIOL Syntheselabor Tempelhofer Weg 11-12 D-10829 Berlin, Germany Phone: (030) 78799455 Fax: (030) 78799499 Email: DNA@TIB-MOLBIOL.de
USA	Genset Corporation 875 Prospect Street, Suite 206 LA JOLLA CA 92037 Phone: +1 619 551 3000 Fax: +1 619 551 3001

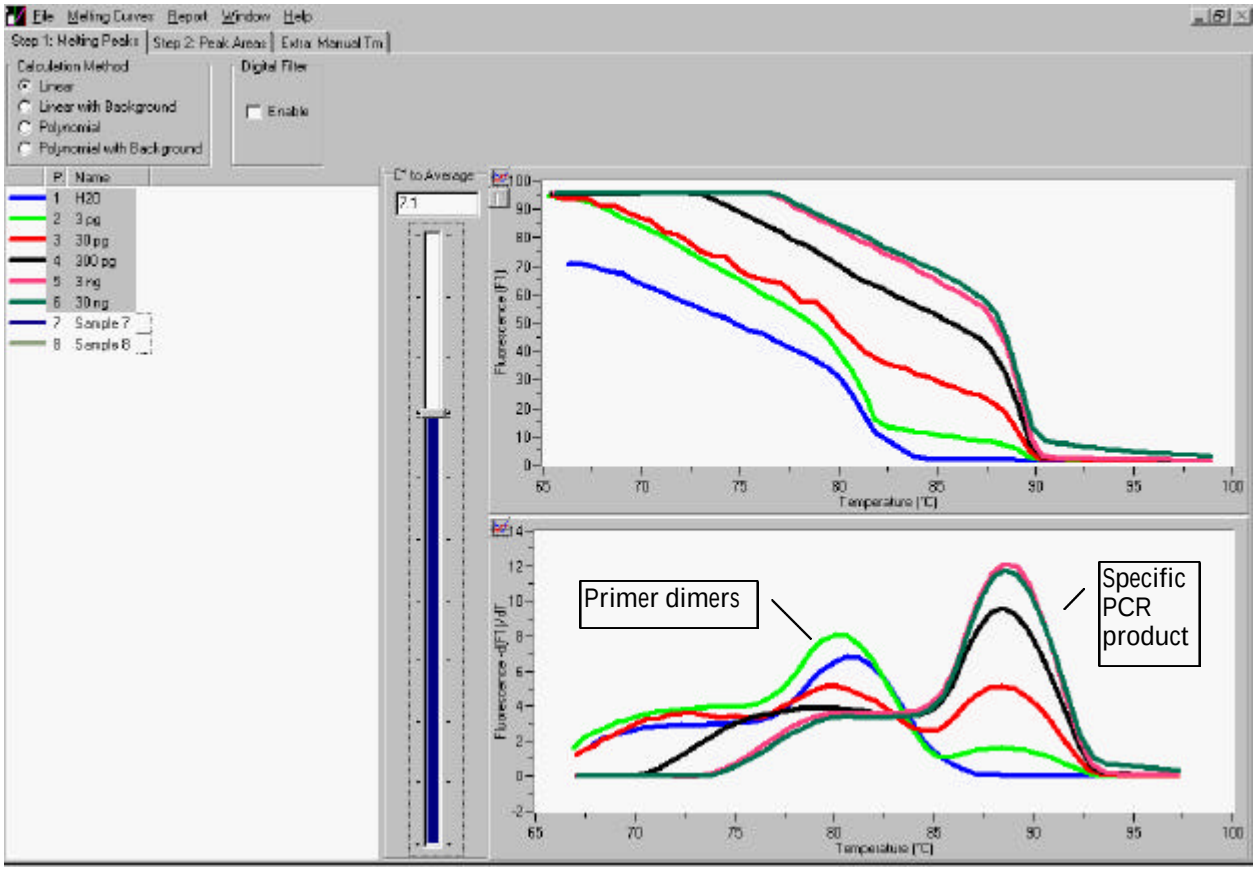
1.4 Melting Curve Analysis

Introduction

To significantly enhance the information obtained from a PCR, the progress of the amplification may be subjected to a melting curve analysis. Amplification products usually melt quickly and thoroughly at a temperature characteristic of the product. Analysis of melting curves with respect to temperature results in sharp, easily detectable melting peaks. Such an analysis may be performed on any PCR that contains SYBR Green I or fluorescent hybridization probes.

Principle

Melting curve analysis is an inversion of the PCR detection method, in which increases in fluorescence are detected during cycling. At the beginning of a melting curve analysis, the reaction is at a low temperature and the fluorescence signal is high. As the temperature steadily increases, the fluorescence will suddenly drop as the reaction reaches the melting point (T_m) of each DNA fragment. As shown in the picture below, a graphical evaluation of melting behavior [plot of the first negative derivative (-dF/dT) of the fluorescence vs. temperature] reveals that pure, homogeneous PCR products produce a single, sharply defined melting curve with a narrow peak. In contrast, primer-dimers melt at relatively low temperatures and have broader peaks.



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Advantages

If the melting temperatures of the desired product and the primers are known, the fluorescence signal may be measured during each cycle at a temperature just above the T_m of the primer-dimers, but below the T_m of the specific PCR product. Such measurements eliminate detection of nonspecific fluorescence.

Applications

Melting curve analysis with the LightCycler instrument allows:

- Use of SYBR Green I to identify and characterize PCR products by their characteristic melting behavior,
- More specific analysis of PCR reactions with SYBR Green I,
- Identification and differentiation of multiple specific PCR products (Multiplex PCR) with SYBR Green I,
- Genotyping and mutation analyses with hybridization probes.

2. Factors that Influence LightCycler PCR Results

Introduction Although PCR is a simple concept, successful PCR depends upon a number of interrelated factors. This section will provide an overview of these factors.

In this Section The topics covered in this section include:

Topic	See Page
Template Preparation	C 31
Preventing Contamination during PCR Setup	C 40
Optimizing the Components of the Reaction Mix	C 41
Optimizing and Troubleshooting the Overall Reaction	C 53

2.1 Template Preparation

Introduction

Every successful PCR depends on the quality of the nucleic acid template that contains the target sequence(s). As a plethora of template preparation methods exist, we cannot review all the available methods here. Instead, we describe a selection of nucleic acid isolation and purification kits from Roche Molecular Biochemicals that our tests have shown to yield reliable LightCycler templates.

Maximum Amount of Nucleic Acid Template in a LC PCR

Based on our experience, we recommend using no more than the following amounts of template in each LightCycler assay:

- 50 ng genomic DNA (for SYBR Green I assays), or
  500 ng genomic DNA (for assays with hybridization probes)
- 1 ng plasmid DNA
- 500 ng total RNA
- 10 ng of mRNA

Requirements for Nucleic Acid Template Preparation

A good nucleic acid purification product should:

- Allow preparation of intact DNA or RNA from numerous biological sources,
- Prepare nucleic acid from both large and small samples,
- Guarantee complete removal of nucleic acids and low molecular weight components that may inhibit or interfere with PCR,
- Not expose the user to chemical hazards (e.g., phenol), and
- Process a large number of samples quickly and conveniently, e.g., by avoiding time-consuming centrifugation and/or precipitation steps.

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Main Stages of Nucleic Acid Template Preparation

Methods used to prepare nucleic acids can be divided into two major stages:

1. *Extraction of the nucleic acids from the biological material.*
   This requires cell lysis, nuclease inactivation, and removal of cellular debris. Techniques include mechanical disruption (grinding, homogenization, or hypotonic lysis), chemical treatment (detergent lysis, chaotropic agents, thiol reduction), or enzymatic digestion (proteinase K treatment).
2. *Purification of the nucleic acids from the prepared extracts.*
   Approaches include extraction/precipitation, chromatography, centrifugation, electrophoresis, affinity separation, or combinations of these techniques.

Note: The combined effectiveness of these two stages determines the reliability, template quality, and convenience of a given protocol.

PCR Inhibitors Present in Mammalian Blood

Blood preparations contain several compounds that can inhibit PCR greatly, including:

- Porphyrin compounds derived from the heme group.
- Anticoagulants such as citrate, EDTA, and heparin.
  Caution*:* As little as 0.05 units of heparin per reaction will suppress PCR.

Note: To eliminate these PCR inhibitors from blood preparations, use Roche Molecular Biochemicals kits to prepare template nucleic acids from blood.

Further Reading

For detailed information on our complete product line for nucleic acid purification, please refer to our manual *Nucleic Acid Isolation and Purification* and the current version of our Biochemicals Catalog. Both publications are available free of charge from your local Roche Molecular Biochemicals representative.

Note: In addition, we regularly publish the results of ongoing applications research, as well as customer protocols in the Roche Molecular Biochemicals *BIOCHEMICA* journal and on our Web page (http://biochem.roche.com).

2.1.1 Selection Guide for LightCycler Template Preparation

Overview

Use the following table to select a product according to the type of nucleic acid you want to purify:

Nucleic Acid	Recommended Kit	See Page
Genomic DNA	High Pure PCR Template Preparation Kit	C 34
Viral nucleic acid	High Pure Viral Nucleic Acid Kit	C 36
Total RNA	High Pure RNA Isolation Kit	C 37
mRNA (from blood)	mRNA Isolation Kit for Blood/Bone Marrow	C 38
mRNA (from cultured cells)	mRNA Isolation Kit	C 39

Principles behind the Kits

The kits in the selection guide above use the following nucleic acid purification methods (principles):

Recommended Kit	Purification Method
High Pure PCR Template Preparation Kit	Silica Adsorption
High Pure Viral Nucleic Acid Kit	Silica Adsorption
High Pure RNA Isolation Kit	Silica Adsorption
mRNA Isolation Kit for Blood/Bone Marrow	Affinity Purification
mRNA Isolation Kit	Affinity Purification

High Pure Technology (Silica Adsorption)

High Pure technology, available exclusively from Roche Molecular Biochemicals, relies on the tendency of nucleic acids to adsorb to silica (glass) in the presence of chaotropic salts [e.g., sodium iodide, guanidine thiocyanate, or guanidine hydrochloride]. Variations of this technology, e.g., changes in the ionic strength and pH of the kit buffers, allow different High Pure Kits to extract different nucleic acids from multiple sources.

Note: Since strong chaotropic salts also inactivate intracellular nucleases, the High Pure extraction buffers help protect the isolated nucleic acids from nucleolytic destruction.

The kits combine chaotropic salt and detergent extraction of sample materials, chromatography on glass fiber fleece, and rapid centrifugation to isolate the desired nucleic acid in an intact, ready-to-use form.

Affinity Purification

The Roche affinity purification kits simplify nucleic acid purification. These kits exploit the hybridization properties of nucleic acids to immobilize particular classes of DNA or RNA on insoluble particles. For mRNA isolation, the kits use biotin-labeled oligo(dT) to capture poly(A)$^+$ mRNA. The hybridized mRNA is then immobilized on streptavidin-coated particles. Thereafter, the particles are separated from surrounding impurities by repeated washing steps and magnetic separation. The magnetic separation step replaces time-consuming centrifugation, organic extraction, and phase separation steps.

2.1.2 Preparing Genomic DNA with the High Pure PCR Template Preparation Kit

Application

In our tests, the High Pure PCR Template Preparation Kit (Cat. No. 1 796 828) prepared suitable genomic LightCycler DNA templates from cultured cells or human whole blood treated with anticoagulants (EDTA, citrate, or heparin).
Note: For conventional PCR, the kit can also prepare genomic DNA templates from buffy coat, tissue, mouse tail, yeast, gram positive/gram negative bacteria, or formalin-fixed, paraffin-embedded tissue sections. For a detailed protocol, see the package insert at our Web site (http://biochem.roche.com/pack-insert/1796828.pdf), or refer to our *Nucleic Acid Isolation and Purification Manual.*

Starting Sample

- Cultured cells: Start with 10 to 10^6 cells.
- Human whole blood: Start with 1 to 1000 µl.

Modifications of Standard Protocol

In order to produce template DNA suitable for LightCycler analysis from heparinized blood, modify the standard High Pure PCR Template Preparation Kit protocol to include an additional, heparin-removing wash step, as follows:

- Follow the standard protocol (from pack insert) through Step 5 (discard flowthrough).
- After Step 5, add 500 µl Wash Buffer I from the High Pure RNA Isolation Kit (Cat. No. 1 828 665) to the upper reservoir of the Filter Tube, then centrifuge the tube assembly for 1 min at 8000 x *g*. This step removes heparin components that can inhibit PCR.
- Discard the flowthrough, reassemble the tube assembly, and resume the standard kit protocol with Step 6 (wash step). Perform the two wash steps recommended in the standard protocol.

Yield

Depending on the amount of starting material, yield of DNA prepared with the kit is between 1 and 20 µg of pure DNA per sample.

Elution Volume

Standard elution volume is 200 µl. However, to increase the concentration of template, you may reduce the elution volume to 50 µl (for maximum amplification sensitivity).

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2.1.2 Preparing Genomic DNA with the High Pure PCR Template Preparation Kit, Continued

Amount of Eluate per PCR Assay

In general, use 5 µl eluate (from a total of 50 µl) in a final volume of 20 µl for LightCycler PCR.
Note: Samples containing 1–10 µl of eluate have been used successfully in the LightCycler.

Amount of DNA per Assay

- For cultured cells, start with DNA from:
  - 1 to 10^4 cells/PCR for SYBR Green I analysis.
  - 1 to 10^5 cells/PCR for analysis with hybridization probes.
    Caution: Do not use DNA equivalent to more than 10^4–10^5 cells/PCR since DNA from larger samples causes excessive background fluorescence or inhibition of PCR.
- For human whole blood, start with DNA from the equivalent of 0.005–100 µl blood/PCR.
  Note: If the calculated sample volume would be <1 µl, dilute master lysate appropriately and use 1–5 µl of diluted lysate as sample.
  Caution: If using DNA from 200 µl blood or more, do not use more than 30% of the sample eluate in PCR.

Sensitivity of PCR

After a LightCycler run, the amplified signal from as little as 2 genome equivalents of starting DNA (from cultured cells) is detectable.

2.1.3 Isolating Viral Nucleic Acid with the High Pure Viral Nucleic Acid Kit

Application	In our tests, the High Pure Viral Nucleic Acid Kit (Cat. No. 1 858 874) isolated suitable viral LightCycler templates from serum, plasma, and cell culture supernatant. ***Note:*** For conventional PCR, the kit can also isolate viral nucleic acids from whole cells. For a detailed protocol, see the package insert at our Web site (http://biochem.roche.com/pack-insert/1858874.pdf), or refer to our *Nucleic Acid Isolation and Purification Manual.*
Starting Sample	• The recommended amount of starting material (serum, plasma, or cell culture supernatant) is 200–600 µl. • The initial concentration of virus in the sample should be $>10^3$ virus particles/ml.
Modifications of Standard Protocol	In order to produce template DNA suitable for LightCycler analysis from heparinized blood, modify the standard High Pure Viral Nucleic Acid Kit protocol to include an additional, heparin-removing wash step, as follows: • Follow the standard protocol (from pack insert) through Step 5 (discard flowthrough). • After Step 5, add 500 µl Wash Buffer I from the High Pure RNA Isolation Kit (Cat. No. 1 828 665) to the upper reservoir of the Filter Tube, then centrifuge the tube assembly for 1 min at 8000 x *g*. This step removes heparin components that can inhibit PCR. • Discard the flowthrough, reassemble the tube assembly, and resume the standard kit protocol with Step 6 (wash step). Perform the two wash steps recommended in the standard protocol.
Elution Volume	Standard elution volume is 50 µl.
Amount of Eluate per PCR Assay	In general, use 5 µl eluate (from a total of 50 µl) in a final volume of 20 µl for LightCycler PCR. ***Note:*** Samples containing 1–10 µl of eluate have been used successfully in the LightCycler.
Amount of Nucleic Acid per Assay	LightCycler PCR can detect nucleic acid from a starting sample that contains 20 to $>10^4$ virus particles/ml.
Sensitivity of PCR	After a LightCycler RT-PCR run, the amplified signal from as little as 20 virus particles/sample is detectable.

2.1.4 Isolating Total RNA with the High Pure RNA Isolation Kit

Application	In our tests, the High Pure RNA Isolation Kit (Cat. No. 1 828 665) isolated suitable LightCycler RNA templates from cultured cells and human whole blood. ***Note:*** For conventional PCR, the kit can also isolate total RNA from yeast, gram positive/gram negative bacteria. For a detailed protocol, see the package insert at our Web site (http://biochem.roche.com/pack-insert/1828665.pdf), or refer to our *Nucleic Acid Isolation and Purification Manual.*
Starting Sample	• Cultured cells: Start with 10 to 10^5 cells. • Human whole blood: Start with 1 to 1000 µl.
Yield	Depending on the type and amount of starting material, the yield of RNA prepared with the kit is ≥15 µg RNA per sample.
Elution Volume	Standard elution volume is 50–100 µl.
Amount of Eluate per Assay	In general, use 5 µl eluate (from a total of 50 µl) in a final volume of 20 µl for LightCycler RT-PCR. ***Note:*** Samples containing 1–10 µl of eluate have been used successfully in the LightCycler.
Amount of RNA per Assay	• For cultured cells, start with RNA from 1 to 10^4 cells/RT-PCR. ***Caution:*** Do not use RNA equivalent to more than 10^5 cells/RT-PCR since RNA from larger samples will inhibit the reaction. • For human whole blood, start with RNA from the equivalent of 0.05–100 µl blood/RT-PCR. ***Note:*** If the calculated sample volume would be <1 µl, dilute master lysate appropriately and use 1–5 µl of diluted lysate as sample.
Sensitivity of RT-PCR	Sensitivity of the assay depends on the parameter measured and the level of RNA expression. ***Guideline:*** In general, the amplified signal from as little as 10–50 mRNA copies can be detected.

2.1.5 Isolating mRNA from Blood with the mRNA Isolation Kit for Blood/Bone Marrow

Application	In our tests, the mRNA Isolation Kit for Blood/Bone Marrow (Cat. No. 1 934 333) isolated suitable LightCycler RNA templates from human whole blood treated with anticoagulants (EDTA, citrate, or heparin). ***Note:*** For conventional PCR, the kit can also isolate mRNA from bone marrow aspirate. For a detailed protocol, see the package insert at our Web site (http://biochem.roche.com/pack-insert/1934333.pdf), or refer to our *Nucleic Acid Isolation and Purification Manual.*
Starting Sample	Start with 0.5–1 ml anticoagulant-treated blood. ***Note:*** For detection of rare sequences, increase sample size to 2–5 ml.
Yield	Typical yield is 50–200 ng mRNA/ml blood.
Elution Volume	For 5 ml of blood sample, standard elution volume is 20 µl. ***Note:*** Elution volume depends on amount of starting material.
Amount of Eluate per Assay	In general, use 1-2 µl eluate (from a total of 20 µl) in a final volume of 20 µl for LightCycler RT-PCR.
Amount of RNA per Assay	Start with RNA from the equivalent of 50–100 µl blood/RT-PCR.
Sensitivity of RT-PCR	Sensitivity of the assay depends on the parameter measured and the level of RNA expression. ***Guideline:*** In general, the amplified signal from as little as 10–50 mRNA copies can be detected.

2.1.6 Isolating mRNA from Cultured Cells with the mRNA Isolation Kit

Source	In our tests, the mRNA Isolation Kit (Cat. No. 1 741 985) isolated suitable LightCycler RNA templates from cultured cells. ***Note:*** For conventional PCR, the kit can also isolate mRNA from tissue and total RNA. For a detailed protocol, see the package insert at our Web site (http://biochem.roche.com/pack-insert/1741985.pdf), or refer to our *Nucleic Acid Isolation and Purification Manual.*
Starting Sample	Start with 10^4 to 10^7 cells.
Yield	Typical yield is 0.3–25 µg mRNA/10^7 cells.
Elution Volume	For 10^7 cells, standard elution volume is 25 µl. ***Note:*** Elution volume depends on the amount of starting material.
Amount of Eluate per Assay	In general, use 1-2 µl eluate (from a total of 25 µl) in a final volume of 20 µl for LightCycler RT-PCR.
Amount of RNA per Assay	Start with mRNA from the equivalent of 10^3 to 10^6 cells/RT-PCR.
Sensitivity	Sensitivity of the asssay depends on the parameter measured and the level of RNA expression. ***Guideline:*** In general, the amplified signal from as little as 10–50 mRNA copies can be detected.

2.2 Preventing Contamination during PCR Setup

Introduction

The ability of the Polymerase Chain Reaction to amplify a single molecule means that even trace amounts of foreign DNA could serve as template, resulting in amplification of the wrong target DNA (false positives).
Possible sources for DNA contamination are outlined below:

- Laboratory benches, equipment, and pipetting devices which can be contaminated by previous DNA preparations, by plasmid DNA, or by purified restriction fragments.
- Products from previous PCR amplifications.
- Cross-contamination between samples.

Laboratory Facilities

At a minimum, set up physically separated working places and pipettes for

- Template preparation before PCR,
- PCR itself, and if desired,
- Post-PCR analysis.

Preparation of Reagents

- Use sterile, aerosol-resistant pipette tips (Aerosol-Resistant Pipette Tips, Cat. No. 1 667 068) and a dedicated (used only for PCR) set of positive displacement pipettes.
- In general, use sterile equipment and laboratory practice; always wear fresh gloves when working in the PCR area. Change gloves frequently, especially if you suspect they have become contaminated with DNA solutions.
- Have your own set of PCR reagents and solutions, and store these reagents in small aliquots.
- When preparing PCR reagents, always use new and/or sterilized glassware, plasticware and pipettes that have never been used to prepare DNA.
- Autoclave all reagents and solutions that can be autoclaved without affecting their performance.
  Note: Primers, dNTPs, and Taq DNA polymerase should not be autoclaved.

Setting Up a PCR

- To avoid carry-over contamination, do not pipette from stock solutions. We recommend transferring the required working solutions into a separate, new tube.
- When pipetting DNA, avoid creating aerosols that could carry contaminants.
- Always include control reactions, for example, a negative or “no DNA” control that contains all of the reaction components except the DNA template.

2.3 Optimizing the Components of the Reaction Mix

Introduction

This section will provide an overview of reaction mix components that affect PCR performance. You will learn more about:

Topic	See Page
Taq DNA Polymerase	C 42
PCR Primer Design	C 43
$MgCl_2$	C 46
Deoxynucleotides (dNTPs)	C 47
Carry-over Prevention with UNG	C 48

2.3.1 Taq DNA Polymerase

Source	Taq DNA polymerase is isolated from the thermophilic eubacterium *Thermus aquaticus* BM.
Size and Activity	Taq DNA polymerase has a single polypeptide chain with a molecular weight of approx. 95 kDa. It is a highly processive 5′–3′ DNA polymerase, which has 5′–3′ exonuclease activity, but lacks 3′–5′ exonuclease (proofreading) activity. The enzyme is most active at a pH of approx. 9 (adjusted at 20°C), and temperatures around 75°C.
Synthesis Rate	Taq DNA polymerase adds an average of 50 nucleotides per second at 75°–80°C.
Thermal Stability	Taq DNA polymerase has a half-life of up to 40 minutes at 95°C. However, the half-life decreases to approx. 5 minutes when the temperature is raised to 100°C.
$MgCl_2$	Taq DNA polymerase prefers Mg^{2+} as cofactor. The standard magnesium concentration for PCR ranges from 1–5 mM for PCR, and 3–8 mM for one-step RT-PCR.
Use with the LightCycler	Roche Molecular Biochemicals offers Taq DNA polymerase as a component of optimized LightCycler - DNA Master Kits (Cat. Nos. 2 015 099, 2 015 102) and LightCycler - RNA Amplification Kits (Cat. Nos. 2 015 137, 2 015 145).

2.3.2 PCR Primer Design

Introduction

Primers are short, single-stranded synthesized oligonucleotides which serve as starting molecules for the *de novo* synthesis of DNA by Taq DNA polymerase. For PCR, you will need one primer complementary to the 3′ end of the target sequence (sense strand) and one complementary to the 3′ end of the antisense strand.
Successful PCR depends upon the design of optimal primer pairs. Suitable primer pairs may be designed manually or with the aid of commercially available computer software. The main criteria for primer design are:

- Length
- Sequence, and
- Melting temperature.

Primer Length

- We recommend using primers that are 18–24 nucleotides long. Shorter primers do not have enough sequence specificity, while longer primers do not increase the specificity.
- Try to make the two primers equal in length.

GC Content

- The GC content of the primers should range between 40% and 60%.
- Whenever possible avoid an unbalanced distribution of G/C- and A/T-rich domains.
- Try to make the two primers equal in GC percentage.

Sequence

- The most important region for specific priming is the 3′ region of the primer; amplification starts here. In general, these 3′ ends should be free of secondary structures, repetitive sequences, palindromes, and highly degenerate sequences.
- The sequences of the two primers should not be complementary to each other, especially at their 3′ ends (so primer-dimers will not form).

Melting Temperatures

- The melting temperature (T_m) of a primer that is bound to target DNA is the temperature at which 50% of the primer-target hybrids are single-stranded.
- The T_m depends mainly on primer sequence and buffer composition. The GC content affects the T_m more than the length of the primer.
- Most primers should have T_ms between 55°C and 65°C. Primers that are used together should have a similar T_m value.

Continued on next page

2.3.2 PCR Primer Design, Continued

Melting Temperatures - Formula

Several programs and algorithms, based on empirical and theoretical data, can be used to determine primer T_ms and optimal annealing temperatures.
Note: Calculated T_ms are poor predictors of the optimum annealing temperature, therefore the annealing temperature of the primers should be determined experimentally. For initial experiments, set the annealing temperature 5°C below the calculated T_m.
Caution: Optimum annealing temperatures may be below or above the calculated T_m. A simple formula for calculating the T_m of manually designed primers is:

T_m = 2°C (A+T) + 4°C (G+C) (Thein and Wallace, 1986)

Primer Design Software

Computer programs simplify the complex task of designing PCR primers. Many of these programs are available for free from World Wide Web Sites. Here are some examples:

Software	Operating systems	Available from*
CODEHOP	On-line	http://blocks.fhcrc.org/codehop.html
DegenDesigner	UNIX	ftp anonymous evolution.bchs.uh.edu (Directory/pub/gene-server/unix)
Primer Design	DOS	ftp anonymous ftp.chemie.uni-marburg.de (Directory/pub/PrimerDesign)
Primer-Master	DOS	ftp anonymous ftp.ebi.ac.uk (Directory/pub/software/dos)
PRIMER-MIT	UNIX. Mac, DOS	ftp anonymous genome.wi.mit.edu (Directory/pub/software/primer)
Primers!	Mac	ftp anonymous wuarchive.wustl.edu (Directory/systems/mac/info-mac/sci/primers-10-installer.hqx)
Primers!	On-line	http://www.williamstone.com/primers
Primer Selection (VGC)	On-line	http://alces.med.umn.edu/VGC.html
Primer Selection (USC)	On-line	http://norp5424b.hsc.usc.edu/genetools.html

Note: We found these sites in a routine search of the Web. We have not evaluated or verified the information contained in them.

Continued on next page

* List compiled in November 1998. Subject to change.

Primer Concentration

Equimolar concentrations of both primers should be used to avoid asymmetric (unbalanced) reaction conditions, which have been shown to decrease PCR fidelity.

The optimum concentration of primers is usually 0.1–0.6 µM. Higher concentrations may promote mispriming and accumulation of nonspecific product. Lower primer concentrations may be exhausted before the reaction is completed, resulting in lower yields of desired products.

Note: For most applications, a primer concentration of 0.5 µM will be sufficient. However, for some systems a higher primer concentration (up to 1 µM) may improve results.

More Information on Primer Design

A detailed discussion on primer design is beyond the scope of this manual. For more detailed information on primer design, consult outside reference sources such as these World Wide Web sites:

For information on	Go to this URL (Web address)*
Primer design software	www.ebi.ac.uk/biocat/biocat.html OR www.chemie.uni-marburg.de/~becker/prim-gen.html OR http://norp5424b.hsc.usc.edu/genetools.html
General hints on primer design	http://alces.med.umn.edu/VGC.html
Designing primers for detecting unknown sequences (degenerate primer design strategies)	www.blocks.fhcrc.org/blocks/help/CODEHOP/manuscript.html OR www.dartmouth.edu/artsci/bio/ambros/protocols/other/koelle/degenerate_PCR.html
Calculating melting point T_m for a given primer	www.chop.edu/research/facil/Napcore/OligoCalc.htm

Note: We found these sites in a routine search of the Web. We have not evaluated or verified the information contained in them.

* List compiled in November 1998. Subject to change.

2.3.3 Magnesium Chloride

Introduction

The concentration of $MgCl_2$ greatly influences efficiency as well as specificity of the amplification reaction.

Concentration in PCR

- The optimum $MgCl_2$ concentration for PCR with the LightCycler varies from 1 to 5 mM. In general, the optimum in the LightCycler is about 0.5 mM higher than the optimum concentration in the corresponding conventional PCR.
- The optimum concentration for RT-PCR with the LightCycler varies from 3 to 8 mM.

Influence on Taq DNA polymerase

Taq DNA polymerase prefers Mg^{2+} as cofactor. In addition, $MgCl_2$ forms soluble complexes with the dNTPs to produce the actual substrate that the polymerase recognizes.

Influence on T_m of DNA

High concentrations of $MgCl_2$ stabilize ds DNA and may lead to:

- Nonspecific primer hybridization, and
- Incomplete denaturation (increase in T_m) of target DNA during PCR cycling.

EDTA

EDTA will bind free Mg^{2+} ions so that they are unavailable for PCR, thus affecting the success of experiments. In general, avoid using EDTA in PCR buffers, or at least, minimize the EDTA concentration.
Caution: Many buffers commonly used in molecular biology, e.g. TE buffer, contain EDTA.

2.3.4 Deoxynucleotide Triphosphates (dNTPs)

Introduction	The concentration of deoxynucleoside triphosphates (dNTPs) can affect specificity as well as fidelity (error rate) of PCR.
Concentration for PCR	The optimum concentration for PCR is 200 µM for each nucleotide. ***Note:*** For carry-over prevention with Uracil-DNA Glycosylase (UNG), substitute dUTP for dTTP in the reaction mix. Use 600 µM dUTP instead of 200 µM dTTP (see Section 2.3.5). The LightCycler DNA Master and RNA Amplification Kits already contain optimum amounts of dNTPs and dUTP.
Balanced Concentration of dNTPs	Always use balanced dNTP solutions (i.e., containing equal concentrations of all four dNTPs) to minimize polymerase error rate. Imbalanced dNTP mixtures will reduce Taq DNA polymerase fidelity.
dNTP and Magnesium Concentration	If you increase the concentration of dNTPs, you must also increase the Mg^{2+} concentration. Increases in dNTP concentration reduce free magnesium, thus interfering with polymerase activity and decreasing primer annealing.

2.3.5 Carry-over Prevention with Heat-labile Uracil DNA Glycosylase (UNG)

Introduction

PCR can amplify a single molecule over a billionfold. Thus, even miniscule amounts of a contaminant can be amplified and lead to a false positive result. Such contaminants are often products from previous PCR amplifications (carry-over contaminants).
Carry-over contaminants can cause enormous problems in routine amplifications that use virtually the same set of primer pairs or primer combinations. Researchers have, therefore, developed methods to avoid carry-over contamination. One simple and effective procedure uses heat-labile Uracil-DNA Glycosylase (Cat. No. 1 775 367).

Principle of UNG Carry-over Prevention

When used as described in the table below, Uracil DNA Glycosylase (UNG) degrades chemically modified PCR products from previous amplifications, but does not degrade native templates (DNA or RNA) or primers.

Stage	Description
1	Substitute dUTP for dTTP during PCR amplification.
2	Prior to the next amplification, treat every PCR sample with heat-labile UNG; treatment leads to apyriminic polynucleotides in contaminating carry-over products.
3	During subsequent PCR, cleave these apyriminic polynucleotides at high temperatures during the initial denaturation step; cleaved polynucleotides can no longer serve as PCR templates.

Source and Storage of UNG

Heat-labile Uracil-DNA Glycosylase is isolated from the marine bacterium BMTU 3346; it is supplied at a concentration of 1 U/µl in storage buffer. This stock solution is stable at –20°C.
Note: The enzyme will lose its activity at elevated temperatures in buffers that lack stabilizers (e.g., PCR buffer).

Specificity

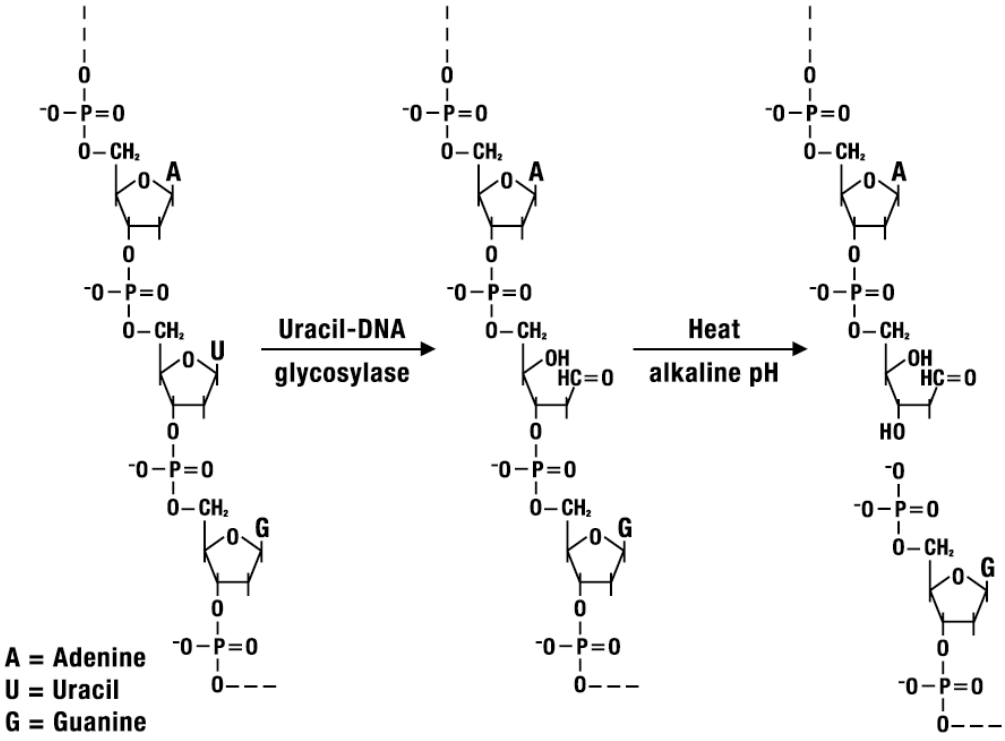
Uracil-DNA Glycosylase catalyzes the removal of uracil from single- and double-stranded DNA that has been synthesized in the presence of dUTP. The apyriminic sites formed by UNG are susceptible to cleavage by heat under alkaline conditions (see the schematic diagram on the next page).
Note: The enzyme is active on both ss- and ds-DNA, but is inactive on RNA and native, uracil-free DNA. The enzyme does not require metal ions and is therefore fully active in the presence of EDTA.

Continued on next page

2.3.5 Carry-over Prevention with Heat-labile Uracil DNA Glycosylase (UNG), Continued

Schematic Representation of UNG Activity

The diagram below shows the hydrolysis of uracil-glycosidic bonds at U-DNA sites and the subsequent cleavage of those apyriminic sites by alkaline pH conditions and heat.



Continued on next page

2.3.5 Carry-over Prevention with Heat-labile Uracil DNA Glycosylase (UNG), Continued

Brief Application Protocol

To use UNG to prevent carry-over contamination, do the following:

Step	Action
1	Add 1 unit heat-labile Uracil-DNA Glycosylase (Cat. No. 1 775 367) per 20 µl LightCycler reaction mix (PCR and RT-PCR). ***Note:*** This amount of enzyme will digest approx. 10^5 copies of a contaminating molecule. Higher concentrations of UNG may interfere with the amplification.
2	Incubate the mix for at least 5 min at room temperature. ***Note:*** In our experience, this incubation is usually completed in the time it takes to set up a PCR run. However, if you are processing very few samples, be careful to allow at least 5 min incubation time.
3	<table><tr><th>IF...</th><th>THEN...</th></tr><tr><td>You run a PCR</td><td>Start your PCR run as usual with a prolonged initial denaturation step, e.g., 2 min at 94°C, to heat-inactivate UNG.</td></tr><tr><td>You run an RT - PCR</td><td>Start the reverse transcription step without denaturation (because the UNG is inactive at temperatures >45°C). Add a high temperature denaturation step (to heat-inactivate UNG) between the reverse transcription step and the PCR.</td></tr></table>

Influence on T_m

The presence of UNG in a reaction mix affects the melting behavior of DNA and lowers the T_m of PCR products. If you are performing melting curve analysis, take this effect into account when setting the experimental parameters. The melting behavior of each individual amplicon should be examined in the presence of UNG.

2.3.6 Hot Start

Introduction

Primer-dimers are the product of nonspecific annealing and primer elongation events. These events take place as soon as PCR reagents are combined, even if reagents are mixed at room temperature. Even if one primer is elongated by only one false nucleotide, this primer may significantly enhance nonspecific amplification.
During PCR, formation of primer-dimers competes with formation of specific PCR product, leading to reduced amplification efficiency and a less successful PCR.
To prevent this nonspecific amplification, use the "Hot Start" method. In this method, at least one of the essential reaction components is separated from the reaction mixture (or blocked/inhibited) until the temperature in the tubes has exceeded the optimal primer annealing temperature.
Note: The formation of primer-dimers is strongly dependent on the sequence of the primers, so if you have already optimized the sequence of your primers, the Hot Start technique will not markedly improve your PCR.

Hot Start with the LightCycler

For Hot Start with the new LightCycler system, we recommend using a commercially available anti-Taq DNA polymerase antibody [e.g., TaqStart Antibody from Clontech (Cat. No. 5400-1, 5400-2)]. This antibody keeps the polymerase inactive until the temperature rises above 70°C. The antibody is inactivated by the same heating step that denatures the target DNA.
Note: The small LightCycler capillaries prevent the use of conventional, manual or wax Hot Start methods.

Continued on next page

How to Use the Anti-Taq Antibody

To use the anti-Taq antibody (e.g., TaqStart Antibody) in a LightCycler PCR, do the following:

Step	Action
1	For each 20 µl reaction (final reaction volume), do one of the following: **IF...** / **THEN...** You use SYBR Green I for detection → Add 0.16 µl of the concentrated TaqStart Antibody solution to 2 µl LightCycler-DNA Master SYBR Green I. You use hybridization probes for detection → Add 0.32 µl of the concentrated TaqStart Antibody solution to 2 µl LightCycler-DNA Master Hybridization Probes.
2	Incubate the mixture for 5 min at room temperature.
3	Add primers, additional $MgCl_2$ (if required), H_2O, the hybridization probe pair (if required), and the DNA template. ***Note:*** The final volume will be 20 µl.
4	Set the initial denaturation time for 1 min at 95°C, and start cycling as usual.

2.4 Optimizing the Overall Reaction

Introduction

This section describes a systematic optimization strategy that will help you easily optimize PCR.
Note: See Section 2.4.1 for a detailed overview of how to troubleshoot the LightCycler assays.

What Needs Optimizing?

To check PCR performance, examine four parameters:

1. **Cycle threshold**: Examine the recorded progress of the run. An efficient amplification reaction will reach the log-linear phase early in the cycle program.
2. **Purity of product**: If using SYBR Green I dye to monitor the reaction, perform a melting curve analysis after amplification to determine whether by-products such as primer-dimers have formed.
3. **Peak heights** (only with SYBR Green I dye): If the amplification reaction does not work properly, the height of the melting peaks will be lower than expected.
4. **Appearance of product on gel**: A successful amplification will usually produce a single bright band on the gel.

Systematic Optimization

If the result of the PCR is not as good as expected, optimize the following parameters in the order given below:

Step	Action
1	Estimate the annealing temperature. Optimum annealing conditions may occur at temperatures up to 5°C lower or 15°C higher than the calculated T_m of the primers.
2	Vary the Mg^{2+} concentration in the experiment; run parallel reactions containing 2, 3, and 4 mM Mg^{2+}. ***Note:*** Raise the Mg^{2+} concentration to 8 mM for RT-PCR applications.
3	Recalculate the time required for extension by assuming the processivity of the polymerase is 25 nucleotides per second.
4	Increase the annealing time.

Chapter D
Applications and Protocols

1. Applications and Protocols

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2. General PCR with the LightCycler

Introduction to LightCycler Applications

The LightCycler instrument combination provides great flexibility to users. To illustrate, we describe in this chapter several LightCycler applications developed by Roche Molecular Biochemicals. Some are designed to analyze any PCR product, regardless of sequence. Others use combinations of fluorescent dyes and oligonucleotide probes to detect specific sequences.
Note: Many of these applications can be completed (including sample preparation, amplification, and data analysis) in less than one hour.

Different Detection Formats

PCR products may be detected with either of two different fluorescence procedures. SYBR Green I dye binds to all double-stranded DNA molecules regardless of their sequence and produces fluorescence proportional to the amount of amplified DNA (see section Chapter C, Section 1.3.1). Alternatively, oligonucleotides coupled to suitable fluorophores form sequence-specific hybridization probes, which can detect and quantify a specific amplicon (see Chapter C, Section 1.3.2).
Note: Examples of both procedures are given in this chapter.

2.1 SYBR Green I Format: Detection and Melting Curve Analysis of the Human β-Globin Gene

2.1.1 Test Overview

Overview

The procedure below describes the amplification of the β-globin gene from human genomic DNA in the presence of the fluorescent dye SYBR Green I, the quantification of the product, and the characterization of the amplicon by melting curve analysis.

Test Principle

The procedure has three main stages.

Stage	Description
1	A fragment of the β-globin gene is amplified from human genomic DNA.
2	After amplification, the fluorescence signals are evaluated with the **Quantification** program in the LightCycler Data Analysis module.
3	Finally, the melting data are analyzed with the **Melting Curve** program.

Sample Material

Human, genomic DNA (vial 1 of the LightCycler - Control Kit DNA, Cat. No. 2 015 129)

Assay Time

The total assay time is approx. 50 minutes. The assay includes the following:

Step	Time
Preparation of samples	15 min
Amplification and melting	25 min
Interpretation of the results	10 min

Additional Material Required

To perform this assay, you will need the following:

- LightCycler - Control Kit DNA (Cat. No. 2 015 129)
- LightCycler - DNA Master SYBR Green I (Cat. No. 2 015 099)
- Standard benchtop microcentrifuge with a rotor for 2.0 ml tubes.
  Note: Centrifuge adapters supplied with the LightCycler Instrument fit into a standard microcentrifuge rotor which holds 2 ml reaction tubes.

2.1.2 Programming the Experimental Protocol

Overview

The Experimental Protocol contains four cycle programs:
- Program 1: Denaturation of the template DNA
- Program 2: Amplification of the target DNA
- Program 3: Melting curve analysis for product identification
- Program 4: Cooling of the instrument

Note: Directly after setting up the last program, you must also set the fluorescence parameters (as described later in this section).

Program 1: Denaturation

Set up the program for denaturation of the DNA template as follows:

Step	Action
1	From the **Front Screen**, click on the **Run** button to access the **Programming Screen**.
2	• Create a new **Experimental Protocol** by clicking on the **New Experiment** button in the **Experiment/RUN/Exit window** (upper left portion of screen). ***Result:*** The computer will now ask for name and path of the experimental protocol to be programmed. • Type a file name for the protocol and specify the file path. • Click **OK**.
3	In the **Cycle Program** list (below the experiment file name in the upper right corner of the screen) click on **Add,** type the name of the program and click **OK.**
4	In the **Cycle Program Data** and **Temperature Targets** windows (center of screen), enter the following program parameters:

Parameter	Value
Cycles	1
Analysis Mode	None
Temperature Targets	**Segment 1** ***Note:*** *Click on* ***INS*** *to insert each new segment!*
Target Temperature (°C)	95
Incubation time (h:min:s)	30
Temperature Transition Rate (°C/s)	20.0
Secondary Target Temperature (°C)	0
Step Size (°C)	0
Step Delay (cycles)	0
Acquisition Mode	None

Result: The program is saved automatically.

Continued on next page

2.1.2 Programming the Experimental Protocol, Continued

Program 2: Amplification

During amplification, the fluorescent signal is measured (acquired) at the end of the elongation phase. Set up the cycling program as follows:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>• To add a new program, click on the last program in the Cycle Program list, then click on Add.
• Type the name of the program, then click OK.</td></tr>
<tr><td>2</td><td>In the Cycle Program Data and Temperature Targets windows, enter the following program parameters:
<table>
<tr><th>Parameter</th><th colspan="3">Value</th></tr>
<tr><td>Cycles</td><td colspan="3">40</td></tr>
<tr><td>Analysis Mode</td><td colspan="3">Quantification</td></tr>
<tr><td>Temperature Targets</td><td>Segment 1</td><td>Segment 2</td><td>Segment 3</td></tr>
<tr><td></td><td colspan="3">Click on INS to insert each new segment!</td></tr>
<tr><td>Target Temperature (°C)</td><td>95</td><td>55</td><td>72</td></tr>
<tr><td>Incubation time (h:min:s)</td><td>0</td><td>5</td><td>10</td></tr>
<tr><td>Temperature Transition Rate (°C/s)</td><td>20.0</td><td>20.0</td><td>20.0</td></tr>
<tr><td>Secondary Target Temperature (°C)</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Step Size (°C)</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Step Delay (cycles)</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Acquisition Mode</td><td>None</td><td>None</td><td>Single</td></tr>
</table>
<u>Result:</u> The program is saved automatically.</td></tr>
</table>

Continued on next page

2.1.2 Programming the Experimental Protocol, Continued

Program 3: Melting Curve Analysis

Set up the program for the melting curve analysis (which will be performed immediately after completion of amplification) as follows:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>• To add a new program, click on the last program in the Cycle Program list, then click on Add.
• Type the name of the program, then click OK.</td></tr>
<tr><td>2</td><td>In the Cycle Program Data and Temperature Targets windows, enter the following program parameters:
<table>
<tr><th>Parameter</th><th colspan="3">Value</th></tr>
<tr><td>Cycles</td><td colspan="3">1</td></tr>
<tr><td>Analysis Mode</td><td colspan="3">Melting Curves</td></tr>
<tr><td>Temperature Targets</td><td>Segment 1</td><td>Segment 2</td><td>Segment 3
<u>Note:</u> Click on INS to insert each new segment!</td></tr>
<tr><td>Target Temperature (°C)</td><td>95</td><td>65</td><td>95</td></tr>
<tr><td>Incubation time (h:min:s)</td><td>0</td><td>10</td><td>0</td></tr>
<tr><td>Temperature Transition Rate (°C/s)</td><td>20.0</td><td>20.0</td><td>0.2</td></tr>
<tr><td>Secondary Target Temperature (°C)</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Step Size (°C)</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Step Delay (cycles)</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Acquisition Mode</td><td>None</td><td>None</td><td>Cont.</td></tr>
</table>
<u>Result:</u> The program is saved automatically.</td></tr>
</table>

Continued on next page

2.1.2 Programming the Experimental Protocol, Continued

Program 4: Cooling

Before removing the rotor from the instrument, you should cool it. Set up the program for the cooling step as follows:

Step	Action
1	• To add a new program, click on the last program in the **Cycle Program** list, then click on **Add**. • Type the name of the program, then click **OK**.
2	In the **Cycle Program Data** and **Temperature Targets** windows, enter the following program parameters: **Parameter** / **Value** Cycles: 1 Analysis Mode: None **Temperature Targets**: **Segment 1** — ***Note:*** *Click on* ***INS*** *to insert each new segment!* Target Temperature (°C): 40 Incubation time (h:min:s): 60 Temperature Transition Rate (°C/s): 20.0 Secondary Target Temperature (°C): 0 Step Size (°C): 0 Step Delay (cycles): 0 Acquisition Mode: None ***Result:*** The program is saved automatically.
3	Save the whole experimental protocol by clicking on the **Save Experiment File** button in the **Experiment/RUN/Exit** window. If you have not already done so, enter a file name.

Setting the Fluorescence Parameters

In the **Display Mode/Fluorimeter Gains** window (upper middle part of screen), set the fluorescence parameters as follows:

- Display Mode: Choose fluorescence channel 1 (**F** 1)
- Set the fluorimeter gains as follows:

Fluorimeter Gain	Value
Channel 1 (F 1)	5
Channel 2 (F 2)	10
Channel 3 (F 3)	10

Continued on next page

2.1.2 Programming the Experimental Protocol, Continued

Naming of Samples

After programming the temperature profiles, type in the sample names and features as follows:

Step	Action
1	Click on the **Edit Samples** button in the **Experiment/RUN/Exit** window (left portion of screen). The **Loading Screen** will appear.
2	The **Loading Screen** contains a **Sample Data** table. In this table, enter the appropriate information for all the samples: • Overwrite the standard settings in the **Sample Name** column (Sample 1, Sample 2, ...) with your sample names. ***Note:*** You can also add the sample names during a PCR run! Refer to Chapter B for more detailed information. • Specify the type of sample (e.g., standard, negative control, unknowns, etc.) in the **Type** column. • In the remaining columns of the table, list all additional sample features [replicates, concentrations (if known), and descriptive notes] • Enter the number of samples in the **Number of Samples** field (lower left corner of window) and click **OK**.
3	Click the **Done** button to return to the **Programming Screen.**

2.1.3 Preparing PCR Samples and Starting the Program

Introduction

Before running the LightCycler Instrument, prepare the samples. Preparation involves four major stages.

Stage	Description
1	Thawing the required solutions.
2	Preparing the sample mixes.
3	Loading the capillaries and placing them into the sample carousel.
4	Placing the sample carousel into the thermal chamber and starting the program.

Thawing the Solutions

Before starting the assay, thaw all the following reagents and store them on ice:

From the ...	Thaw the...
LightCycler - DNA Master SYBR Green I	• LightCycler - DNA Master SYBR Green I (vial 1, green cap) • $MgCl_2$ stock solution (vial 2, blue cap)
LightCycler - Control Kit DNA	• β-Globin Primer Mix (vial 2, white cap) • H_2O, sterile, PCR grade (vial 4, colorless cap)

Setting Up Capillaries

Place LightCycler capillaries into precooled centrifuge adapters. Use one capillary for every sample to be analyzed.
Note: Do not touch the surface of the capillaries with bare fingers. Always wear gloves when handling the capillaries.

Preparing a Dilution Series of the Control

- On the day of the run, prepare a dilution series of the human genomic DNA (LightCycler – Control DNA Kit, vial 1, purple cap, 15 ng/µl) in water (vial 4, colorless cap) as follows:

  1:10 = 1.5 ng/µl
  1:100 = 150 pg/µl
  1:1000 = 15 pg/µl
  1:10 000 = 1.5 pg/µl

- For PCR samples, use 2 µl of each dilution (including the undiluted DNA) as templates.

Continued on next page

2.1.3 Preparing PCR Samples and Starting the Program,
Continued

Negative Control

To prepare a negative control, replace the template DNA with PCR grade water (vial 4, colorless cap) in a reaction mix containing all other reaction components.
Note: Always run a negative control with the samples!

Preparation of Sample Mixes

Prepare PCR samples as follows:
Note: This procedure is designed for a 20 µl standard reaction.

Step	Action
1	In a 1.5 ml reaction tube on ice, prepare enough master mix for all samples to be analyzed. Add the components in the order given below. ***Note:*** The table below lists the amounts of each component needed for one reaction. To determine the total volume of each component needed, multiply the amount in the "Volume" column by (n+1), where n = the number of reactions to be cycled.
2	• Pipet 18 µl master mix into each precooled capillary. • Add 2 µl of the appropriate DNA template (undiluted or pre-diluted).

Component	Volume	Final conc.
H_2O, sterile, PCR grade (vial 4, colorless cap)	11.6 µl	
$MgCl_2$ stock solution (vial 2, blue cap)	2.4 µl	4 mM
β-Globin Primer Mix (vial 2, white cap)	2.0 µl	0.5 µM
LightCycler - DNA Master SYBR Green I (vial 1, green cap)	2.0 µl	1x
Total volume	**18.0 µl**	

Continued on next page

2.1.3 Preparing PCR Samples and Starting the Program, Continued

Preparation of Sample Mixes (continued)

Step	Action
3	• Seal each capillary with a stopper and place the adapter, containing the capillary, into a standard benchtop microcentrifuge. • Centrifuge at 700 x g (3000 rpm in a standard benchtop microcentrifuge) for 5 s. ***Note:*** Arrange the centrifuge adapters so the rotor is in balance, i.e., opposing samples should weigh the same.
4	Place the capillaries in the sample carousel of the LightCycler. ***Note:*** Samples can also be kept in the cooling block at 4°C until they are placed into the sample carousel.

Program Start

Start the run as follows:

Step	Action
1	Place the loaded sample carousel into the thermal chamber.
2	Click the **Run** button. ***Result:*** During the "Seek Process", the LightCycler will find the optimal capillary position for fluorescence detection of each individual sample and then start the programmed run.

End of Experimental Protocol

When the protocol is finished, the LightCycler Software will automatically exit the **Running Screen** and display the collected data on the **LightCycler Data Analysis (LCDA) Front Screen**.

2.1.4 Data Analysis with the Quantification Program

Starting Quantification Analysis

To start data evaluation with the **Quantification Program**, use this procedure:

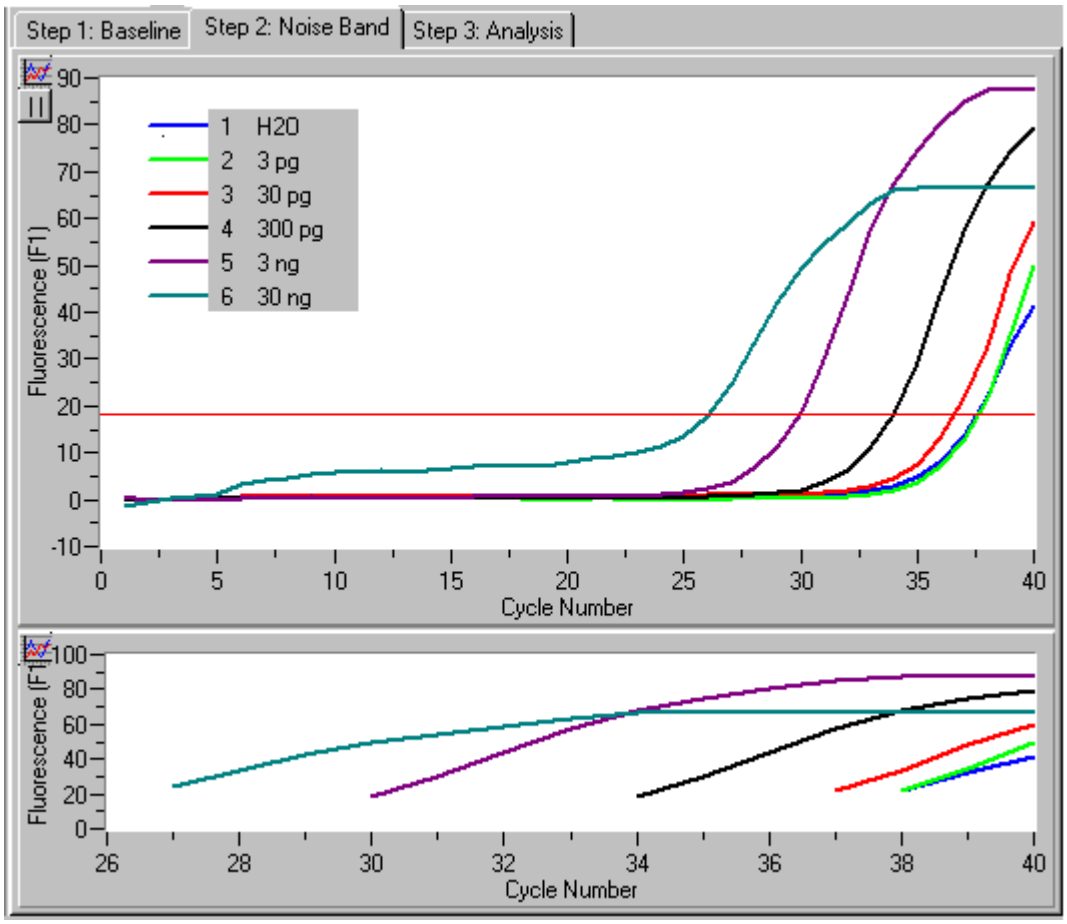
Step	Action
1	Do one of the following: • If the instrument has just finished a run and the **LCDA Front Screen** is displayed, skip Steps 2 and 3 below and go to Step 4. • If no run has been performed, (e.g. if you want to analyze data collected during a previous run), go to Step 2 below.
2	• Click on the **Data Analysis** button on the **LightCycler Front Screen**. • In the window that appears, select "Open" from the **File** menu. ***Result:*** The **Select Data File** window will open.
3	Open the appropriate run file by highlighting a file name and clicking on **Open**. ***Result:*** The **LCDA Front Screen** will appear.
4	Make sure that the amplification segment of the upper temperature chart is selected for analysis. ***Note:*** To select the amplification segment, choose the appropriate (amplification) program segment of the protocol in the **Select a Program** pull down menu.
5	Verify that the fluorescence display mode for the lower graph is **F 1** (in the window to the left of the graph).
6	Enter the **Quantification Screen** by clicking on the **Quantification** button (top center portion of screen).
7	Optimize the data display and analyze the data as described in Section 5 of Chapter B. ***Note:*** See Section 4 of this chapter for more information on the quantification process.

Continued on next page

2.1.4 Data Analysis with the Quantification Program, Continued

Typical Result

The picture below shows quantification data from a typical run of the procedure described in Section 2.1.



Result: The LightCycler SYBR Green I procedure can detect from 30 ng to 3 pg (1 genome equivalent) of human genomic DNA.

2.1.5 Data Analysis with the Melting Curve Program

Overview

The **Melting Curve** program can be used for additional analysis of the experimental data. Because primer-dimers melt at much lower temperatures than specific PCR products, this program can differentiate signals obtained from the specific PCR product from those of primer-dimers.

Selecting the Program

For data evaluation with the **Melting Curve** program, do the following:

Step	Action
1	Return to the **LCDA Front Screen** by minimizing or closing the open window.
2	Choose the Melting Curve program segment of the protocol by clicking on the **Select a Program** button and highlighting the appropriate program segment. ***Result:*** The cursors in the Temp Chart will automatically be set to the beginning and end of the Melting Curve.
3	Click on the **Melting Curve** button (top center portion of screen) to enter the **Melting Curve Analysis Screen**.
4	Verify that fluorescence is set to **F 1** for the y-axis of the lower graph (left lower corner of the graph).
5	Optimize the data display and analyze the melting curve as described in Section 6 of Chapter B.

Determining Melting Temperature

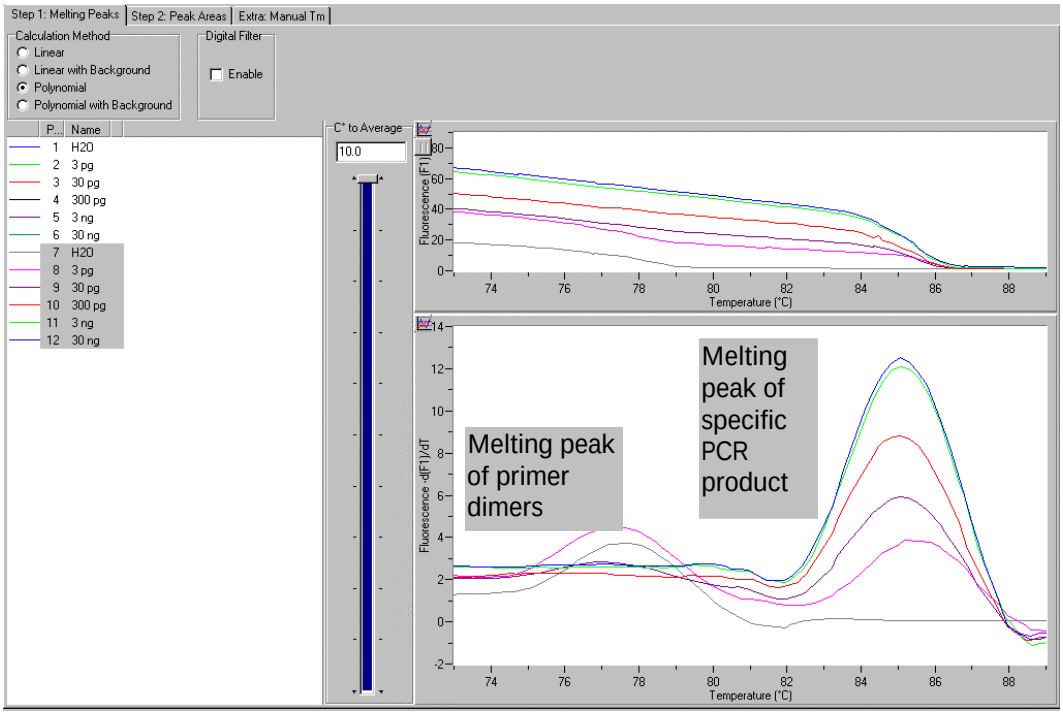
To determine the melting temperature of a product, select the **Extra: Manual Tm** tab at the top of the **Melting Curve Analysis Screen** and use the mouse to move one cursor onto each of the peaks in the "Extra: Manual Tm" section.
Note: The T_m for each product is the highest point on the melting peak.

Continued on next page

2.1.5 Data Analysis with the Melting Curve Program, Continued

Typical Result: Identification of Products

The picture below shows melting curve data from a typical run of the procedure described in Section 2.1.



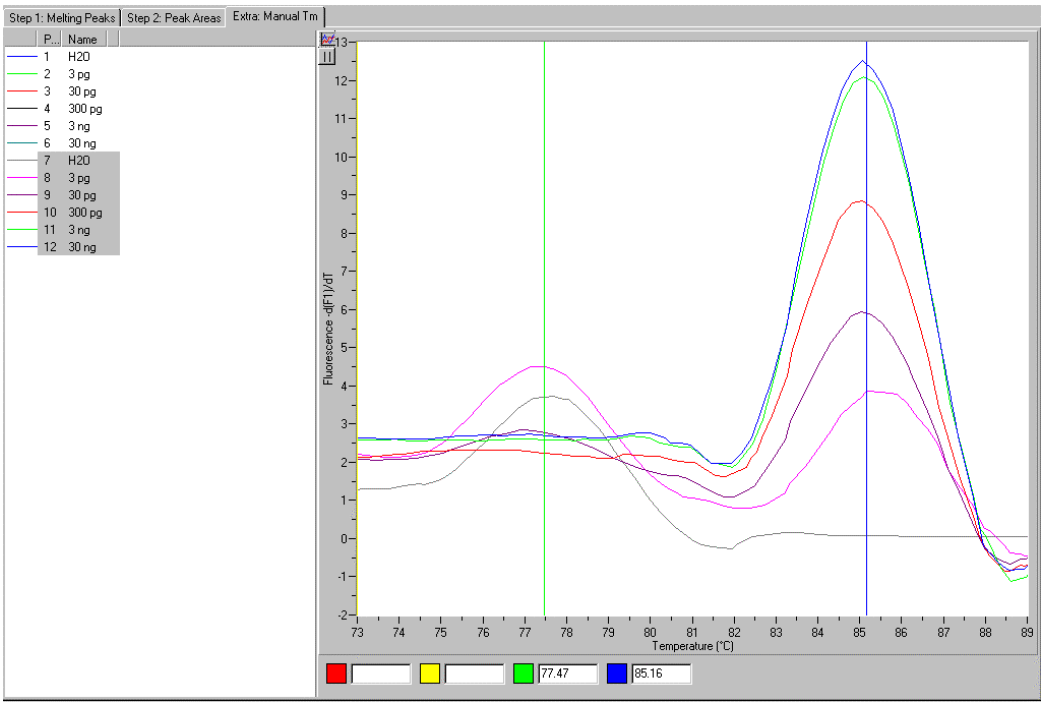
Result: Although significant amounts of primer-dimers are present, the Melting Curve Program can distinguish a fluorescent product-specific signal from only 3 pg (1 genome equivalent) human genomic DNA.

Continued on next page

2.1.5 Data Analysis with the Melting Curve Program, Continued

Typical Result: Determination of Melting Temperature

The picture below shows a determination of melting points in the "Extra: Manual Tm" section of the **Melting Curve Analysis Screen**.
Example: In this run, the T_m (highest point on the melting peak) for the primer-dimer is 77.47°C and the melting peak for the specific product is 85.16°C.



Exit

To exit the **Melting Curve Analysis Screen**, close or minimize the open window and return (automatically) to the **LCDA Front Screen**.

2.2 Hybridization Probes Format: Detection of the Human β-Globin Gene

2.2.1 Test Overview

Introduction

The procedure below describes the amplification of the β-globin gene from human genomic DNA and the quantification of the product with sequence-specific hybridization probes.

Test Principle

The procedure has two main stages.

Stage	Description
1	A fragment of the β-globin gene is amplified from human genomic DNA.
2	After amplification, the fluorescence signals are evaluated using the **Quantification** program.

Sample Material

Human, genomic DNA (vial 1 of the LightCycler - Control Kit DNA, Cat. No. 2 015 129)

Assay Time

The total assay time is approx. 50 minutes. The assay includes the following:

Step	Time
Preparation of samples	15 min
Amplification and melting	25 min
Interpretation of the results	10 min

Additional Material Required

To perform this assay you will need the following:

- LightCycler - Control Kit DNA (Cat. No. 2 015 129)
- LightCycler - DNA Master Hybridization Probes (Cat. No. 2 015 102)
- Standard benchtop microcentrifuge with a rotor for 2.0 ml tubes
  Note: Centrifuge adapters supplied with the LightCycler Instrument fit into a standard microcentrifuge rotor which holds 2 ml reaction tubes.

2.2.2 Programming the Experimental Protocol

Overview

The Experimental Protocol contains three cycle programs:
- Program 1: Denaturation of the template DNA
- Program 2: Amplification of the target DNA
- Program 3: Cooling of the instrument

Note: Directly after setting up the last program, you must also set the fluorescence parameters (as described later in this section).

Program 1: Denaturation

Set up the program for the denaturation step as follows:

Step	Action
1	From the **Front Screen**, click on the **Run** button to access the **Programming Screen**.
2	• Create a new **Experimental Protocol** by clicking on the **New Experiment** button in the **Experiment/RUN/Exit window** (upper left portion of screen). ***Result:*** The computer will now ask for name and path of the experimental protocol to be programmed. • Type a file name for the protocol and specify the file path. • Click **OK**.
3	In the **Cycle Program** list (below the experiment file name in the upper right corner of the screen) click on **Add,** type the name of the program and click **OK.**
4	In the **Cycle Program Data** and **Temperature Targets** windows (center of screen), enter the following program parameters:

Parameter	**Value**
Cycles	1
Analysis Mode	None
Temperature Targets	**Segment 1** ***Note:*** *Click on* ***INS*** *to insert each new segment!*
Target Temperature (°C)	95
Incubation time (h:min:s)	30
Temperature Transition Rate (°C/s)	20
Secondary Target Temperature (°C)	0
Step Size (°C)	0
Step Delay (cycles)	0
Acquisition Mode	None

Result: The program is saved automatically.

Continued on next page

2.2.2 Programming the Experimental Protocol, Continued

Program 2: Amplification

During amplification, the fluorescence signal is measured (acquired) at the end of the annealing phase.
Note: At this stage, the hybridization probes have annealed to their target sequence and a fluorescent signal has been generated, but the Taq polymerase has not yet traveled far enough along the DNA strand to displace the probes.

Set up the cycling program as follows:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>• To add a new program, click on the last program in the Cycle Program list, then click on Add.
• Type the name of the program, then click OK.</td></tr>
<tr><td>2</td><td>In the Cycle Program Data and Temperature Targets windows, enter the following program parameters:
<table>
<tr><th>Parameter</th><th colspan="3">Value</th></tr>
<tr><td>Cycles</td><td colspan="3">45</td></tr>
<tr><td>Analysis Mode</td><td colspan="3">Quantification</td></tr>
<tr><td>Temperature Targets</td><td>Segment 1</td><td>Segment 2</td><td>Segment 3
<u>Note:</u> Click on INS to insert each new segment!</td></tr>
<tr><td>Target Temperature (°C)</td><td>95</td><td>55</td><td>72</td></tr>
<tr><td>Incubation time (h:min:s)</td><td>0</td><td>10</td><td>5</td></tr>
<tr><td>Temperature Transition Rate (°C/s)</td><td>20.0</td><td>20.0</td><td>20.0</td></tr>
<tr><td>Secondary Target Temperature (°C)</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Step Size (°C)</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Step Delay (cycles)</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Acquisition Mode</td><td>None</td><td>Single</td><td>None</td></tr>
</table>
<u>Result:</u> The program is saved automatically.</td></tr>
</table>

Continued on next page

Program 3: Cooling

Before removing the rotor from the instrument, you should cool it. Set up the program for the cooling step as follows:

Step	Action
1	• To add a new program, click on the last program in the **Cycle Program** list, then click on **Add**. • Type the name of the program, then click **OK**.
2	In the **Cycle Program Data** and **Temperature Targets** windows, enter the following program parameters: **Parameter** / **Value** Cycles: 1 Analysis Mode: None **Temperature Targets**: **Segment 1** — ***Note:*** *Click on* ***INS*** *to insert each new segment!* Target Temperature (°C): 40 Incubation time (h:min:s): 60 Temperature Transition Rate (°C/s): 20.0 Secondary Target Temperature (°C): 0 Step Size (°C): 0 Step Delay (cycles): 0 Acquisition Mode: None ***Result:*** The program is saved automatically.
3	Save the whole experimental protocol by clicking on the **Save Experiment File** button in the **Experiment/RUN/Exit** window. If you have not already done so, enter a file name.

Setting the Fluorescence Parameters

In the **Display Mode/Fluorimeter Gains** window (upper middle part of screen), set the fluorescence parameters as follows:

- Display Mode: Choose fluorescence channel 1 (**F** 1)
- Set the fluorimeter gains as follows:

Fluorimeter Gain	Value
Channel 1 (**F** 1)	1
Channel 2 (**F** 2)	10
Channel 3 (**F** 3)	10

Continued on next page

2.2.2 Programming the Experimental Protocol, Continued

Naming the Samples

After programming the temperature profiles, type in the sample names and features as follows:

Step	Action
1	Click on the **Edit Samples** button in the **Experiment/RUN/Exit** window (left portion of screen). The **Loading Screen** will appear.
2	The **Loading Screen** contains a **Sample Data** table. In this table, enter the appropriate information for all the samples: • Overwrite the standard settings in the **Sample Name** column (Sample 1, Sample 2, ...) with your sample names. ***Note:*** You can also add the sample names during a PCR run! Refer to Chapter B for more detailed information. • Specify the type of sample (e.g., standard, negative control, unknowns, etc.) in the **Type** column. • In the remaining columns of the table, list all additional sample features [replicates, concentrations (if known), and descriptive notes]. ***Note:*** For standards that have known template concentrations, you may note those concentrations in the "Concentration" column of the table. This column of the table can only be filled before the run is started! ***Caution:*** Do not designate a negative ("no template") control as a known standard with "**0"** concentration. • Enter the number of samples in the **Number of Samples** field (lower left corner of window) and click **OK**.
3	Click the **Done** button to return to the **Programming Screen.**

2.2.3 Preparing PCR Samples and Starting the Program

Introduction

Before running the LightCycler Instrument, prepare the samples. Preparation involves four major stages.

Stage	Description
1	Thawing the required solutions.
2	Preparing the sample mixes.
3	Loading the capillaries and placing them into the sample carousel.
4	Placing the sample carousel into the thermal chamber and starting the program.

Thawing the Solutions

Before starting the assay, thaw all the following reagents and store them on ice:

From the...	Thaw the...
LightCycler - DNA Master Hybridization Probes	• LightCycler - DNA Master Hybridization Probes (vial 1, red cap) • $MgCl_2$ stock solution (vial 2, blue cap)
LightCycler - Control Kit DNA	• β-Globin Primer Mix (vial 2, white cap) • β-Globin Hybridization Probe Mix (vial 3, yellow cap) • H_2O, sterile, PCR grade (vial 4, colorless cap)

Setting Up Capillaries

Place LightCycler capillaries into precooled centrifuge adapters. Use one capillary for every sample to be analyzed.
Note: Do not touch the surface of the capillaries with bare fingers. Always wear gloves when handling the capillaries.

Preparing a Dilution Series of the Control

- On the day of the run, prepare a dilution series of the human genomic DNA (LightCycler – Control DNA Kit, vial 1, purple cap, 15 ng/µl) in water (vial 4, colorless cap) as follows:
  1:10 = 1.5 ng/µl
  1:100 = 150 pg/µl
  1:1000 = 15 pg/µl
  1:10 000 = 1.5 pg/µl
- For PCR samples, use 2 µl of each dilution (including the undiluted DNA) as templates.

Continued on next page

2.2.3 Preparing PCR Samples and Starting the Program, Continued

Negative Control

To prepare a negative control, replace the template DNA with PCR grade water (vial 4, colorless cap) in a reaction mix containing all other reaction components.
Note: Always run a negative control with the samples!

Preparation of the Sample Mixes

Prepare PCR samples as follows:
Note: This procedure is designed for a 20 µl standard reaction.

Step	Action
1	In a 1.5 ml reaction tube on ice, prepare enough master mix for all samples to be analyzed. Add the components in the order given below. ***Note:*** The table below lists the amounts of each component needed for one reaction. To determine the total volume of each component needed, multiply the amount in the "Volume" column by (n+1), where n = the number of reactions to be cycled.

Component	Volume	Final conc.
H_2O, sterile, PCR grade (vial 4, colorless cap)	9.6 µl	
$MgCl_2$ stock solution (vial 2, blue cap)	2.4 µl	4 mM
β-Globin Primer Mix (vial 2, white cap)	2.0 µl	0.5 µM
β-Globin Hybridization Probe Mix (vial 3, yellow cap)	2.0 µl	• Probe 1: 0.2 µM • Probe 2: 0.4 µM
LightCycler - DNA Master Hybridization Probes (vial 1, red cap)	2.0 µl	1x
Total volume	**18 µl**	

2	• Pipet 18 µl master mix into each precooled capillary. • Add 2 µl of the appropriate DNA template (undiluted or pre-diluted).

Continued on next page

2.2.3 Preparing PCR Samples and Starting the Program,
Continued

Preparation of the Sample Mixes **(continued)**

Step	Action
3	• Seal each capillary with a stopper and place the adapter, containing the capillary, into a standard benchtop centrifuge. • Centrifuge at 700 x g (3000 rpm in a standard benchtop centrifuge) for 5 s. ***Note:*** Arrange the centrifuge adapters, so the rotor is in balance, i.e., opposing samples should weigh the same.
4	Place the capillaries in the sample carousel of the LightCycler. ***Note:*** Samples can also be kept in the cooling block at 4°C until they are placed into the sample carousel.

Program Start

Start the run as follows:

Step	Action
1	Place the loaded sample carousel into the thermal chamber.
2	Click the **Run** button. ***Result:*** During the "Seek Process", the LightCycler will find the optimal capillary position for fluorescence detection of each individual sample and then start the programmed run.

End of Experimental Protocol

When the protocol is finished, the LightCycler Software will automatically exit the **Running Screen** and display the collected data on the **LightCycler Data Analysis (LCDA) Front Screen**.

2.2.4 Data Analysis with the Quantification Program

Starting Quantification Analysis

To start data evaluation with the **Quantification Program**, use this procedure:

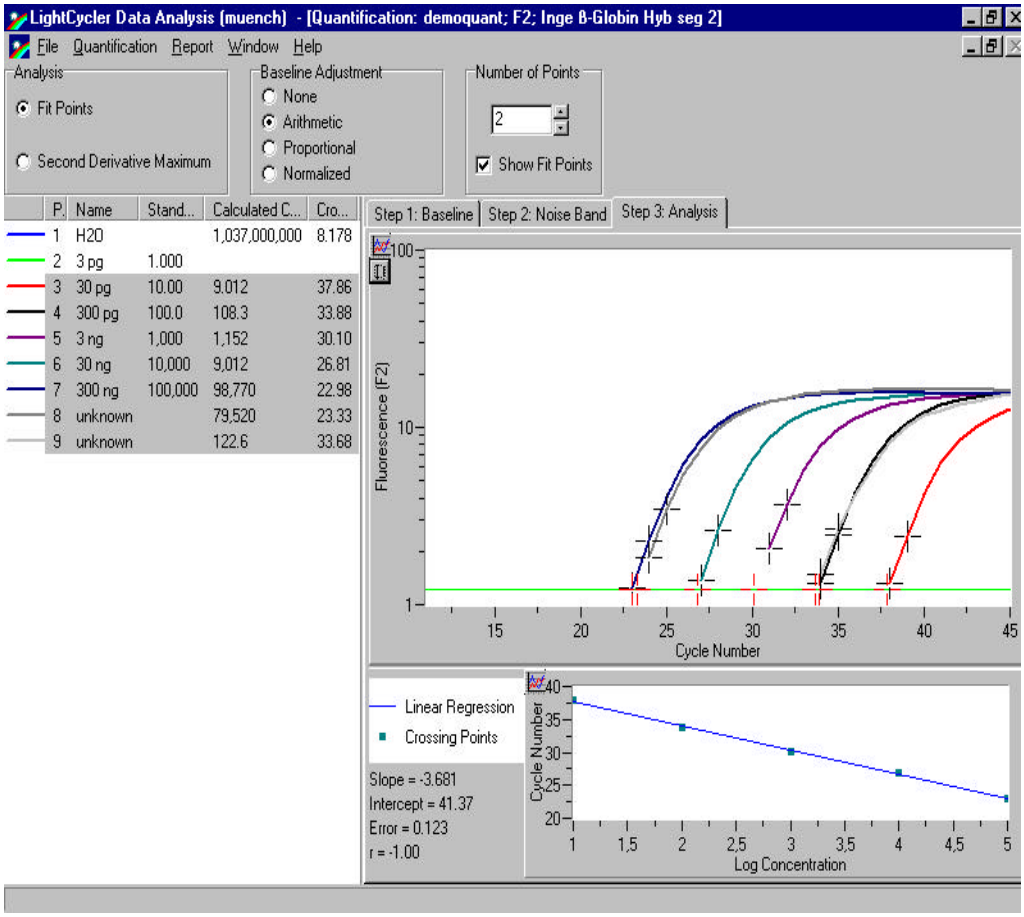
Step	Action
1	Do one of the following: • If the instrument has just finished a run and the **LCDA Front Screen** is displayed, skip Steps 2 and 3 below and go to Step 4. • If no run has been performed, (e.g. if you want to analyze data collected during a previous run), go to Step 2 below.
2	• Click on the **Data Analysis button** on the **LightCycler Front Screen**. • In the window that appears, select "Open" from the **File** menu. ***Result:*** The **Select Data File** window will open.
3	Open the appropriate run file by highlighting a file name and clicking on **Open**. ***Result:*** The **LCDA Front Screen** will appear.
4	Make sure that the amplification segment of the upper temperature chart is selected for analysis. ***Note:*** To select the amplification segment, choose the appropriate (amplification) program segment of the protocol in the **Select a Program** pull down menu.
5	Verify that the fluorescence display mode for the lower graph is **F2/F1** (in the window to the left of the graph).
6	Enter the **Quantification Screen** by clicking on the **Quantification** button (top center portion of screen).
7	Optimize the data display and analyze the data as described in Section 5 of Chapter B. ***Note:*** See Section 4 of this chapter for more information on the quantification process.

Continued on next page

2.2.4 Data Analysis with the Quantification Program, Continued

Typical Result

The picture below shows quantification data from a typical run of the procedure described in Section 2.2. The “Fit Points” method was used for analysis.



Result: The LightCycler Hybridization Probes procedure can detect 30 pg (10 genome equivalents) of human genomic DNA.

3. Reverse Transcription PCR with the LightCycler

Introduction

Gene expression, including both transcriptional and translational processes, and gene regulation are the basis of all cell and tissue functions. These processes are affected in a variety of human disorders. Therefore, in biomedical research, it is often informative to measure the concentration, i.e., the copy number, of a certain target mRNA in normal and pathological samples.

However, any successful mRNA measurement must be capable of detecting very small amounts of RNA. The typical mammalian cell contains approximately 50 pg RNA. Only 2–5% of that total cellular RNA is mRNA. The copy number of a given mRNA target can vary from $>10^4$ copies to less than 1 copy per cell.

It is not surprising, then, that researchers coupled reverse transcription with amplification to produce the RT-PCR method for measuring mRNA. Like PCR, RT-PCR is very sensitive; it is capable of detecting even rare messages in total cell RNA. Also, unlike conventional methods, e.g. northern blotting, *in situ* hybridization, or RNase Protection Assays, RT-PCR is fast, efficient, and very specific.

This section provides a brief overview of the power and potential of RT-PCR assays conducted in the LightCycler. We will describe:

- Basic information on RT-PCR, and
- How to set up One-Step and Two-Step RT-PCR protocols in the LightCycler.

3.1 Basic Information about RT-PCR

Overview

This section gives a brief overview of factors to consider in RT-PCR, including:

- Preparing RT-PCR templates
- Choosing RT-PCR primers,
- Properties of reverse transcriptases, and
- Advantages of One-Step and Two-Step RT-PCR procedures.

Preparing RT-PCR Templates

Cellular ribonucleases (RNases) are ubiquitous, stable, and do not require specific cofactors. Therefore, any successful RNA isolation procedure must, at a minimum, protect the isolated RNA from ribonuclease degradation.
The most successful methods for the isolation of pure, intact RNA combine sample disintegration in the presence of guanidinium thiocyanate and high speed affinity purification of the nucleic acid. Guanidinium thiocyanate inhibits RNases, while affinity purification rapidly isolates the target RNA from almost all other cellular components. Section 2.1 of Chapter C provides a detailed description of such RNA isolation methods, including detailed protocols for the preparation of total RNA or poly(A)$^+$ RNA.

Choosing RT-PCR Primers

Priming affects the size and specificity of the cDNA produced. There are three types of primers that may be used for RT-PCR:

- **Oligo(dT)$_{12-18}$** binds to the poly(A) tail at the 3′ end of mammalian mRNA. This primer can produce full-length cDNA products. Although oligo(dT) priming is often used for conventional Two-Step RT-PCR (see below), it cannot be used in One-Step RT-PCR.
  Caution: Because of secondary structures found in mRNA, oligo(dT)-priming at the 3′ end can lead to a cDNA copy that lacks the 5′ end of the message.
- **Random hexanucleotides** bind to RNA at any complementary site and will lead to partial cDNAs of varying length. Random hexanucleotide priming is often used for conventional Two-Step RT-PCR (see below), but cannot be used in One-Step RT-PCR.
  Note: In contrast to oligo(dT) priming, random hexanucleotide priming may resolve certain problems caused by secondary structures in RNA.
- **Sequence-specific oligonucleotide primers** selectively prime the mRNA of interest.
  Note: The specificity of a Two-Step RT-PCR can be further enhanced by the presence of two different pairs of specific primers, one for the RT-step and the other for PCR. The primer pair for the PCR is then designed to “nest” within (bind inside of) the binding sequence for the first primer pair.

Continued on next page

Properties of Reverse Transcriptases

Reverse transcriptases are RNA-dependent DNA polymerases which catalyze first strand DNA synthesis as soon as a suitable primer with a free 3′ OH end is available. However, reverse transcriptases are also capable of synthesizing a DNA strand complementary to a primed single stranded DNA molecule. Four different well characterized enzymes with reverse transcriptase activity are commercially available from Roche Molecular Biochemicals:

- Two mesophilic viral reverse transcriptases from murine leukemia virus (M-MuLV) and avian myeloblastosis virus (AMV),
- Expand Reverse Transcriptase, a modified M-MuLV RTase (not for sale in the US!), and
- Two thermally active DNA polymerases with reverse transcription activity from *Carboxydothermus hydrogenoformans* (C. therm. polymerase) and *Thermus thermophilus* (Tth).

These enzymes have different biochemical properties (listed in the following table) that determine their suitability for RT-PCR applications.

	M-MuLV		**AMV**	**C. therm. Polymerase**	**Tth**
	wild type	**Expand RT**			
RT activity	+	+	+	+	+
RNase H activity	+	–	+	–	(–)
Cofactor	Mg^{2+}	Mg^{2+}	Mg^{2+}	Mg^{2+}	Mn^{2+}
Temperature Optimum (°C)	37°	42° (up to 50°)	42° (up to 60°)	60°–70°	60°–70°
Thermostability (at 94°C)	–	–	–	–	+
Maximum size of product (in kb)	10	14	12	4	1
RT priming • Hexamer • Oligo(dT) • Sequence-specific	 + + +	 + + +	 + + +	 – + +	 – (–) +

Continued on next page

Advantages of Two-Step and One-Step RT-PCR

In designing an RT-PCR for a specific application, you must first choose which reaction format to use, Two-Step or One-Step RT-PCR. In brief, the characteristics and advantages of the two formats are

- **Two-Step RT-PCR** allows both reverse transcription and PCR to be performed separately, under optimal conditions. This method is especially useful for experiments that require the same RT product to be used for analysis of multiple transcripts.
  Note: In a two-step method, the cDNA synthesis reaction is primed with either random hexamers, oligo(dT), or sequence-specific primers, and performed by a suitable reverse transcriptase (see "Properties of Reverse Transcriptases" above). After reverse transcription, the reverse transcriptase is heat inactivated and an aliquot of the RT reaction (usually 2 µl) is transferred to another tube for PCR. The second tube contains an optimized PCR buffer and a thermostable polymerase. A detailed protocol for Two-Step RT-PCR in the LightCycler is given in Section 3.4 of this chapter.
- **One-Step RT-PCR** allows both cDNA synthesis and PCR amplification to be performed in a single tube with either one enzyme or an enzyme blend. This minimizes both the time required and the chance of sample contamination. This method is especially useful for experiments that require maximum amplification specificity and sensitivity.
  Note: In a one-step method, one set of sequence-specific primers and a single optimized buffer are used for both reverse transcription and PCR. The procedure does not require a sample transfer or the addition of reagents between the two reactions. Detailed protocols for One-Step RT-PCR in the LightCycler are given in Sections 3.2 and 3.3 of this chapter.

3.2 One-Step RT-PCR of TNF-α RNA with SYBR Green I

3.2.1 Test Overview

Introduction

The procedure below describes reverse transcription and subsequent amplification of Tumor Necrosis Factor-α (TNF-α) RNA in a single tube. The reaction is performed in the presence of the fluorescent dye, SYBR Green I, which allows online analysis of the reaction.

Test Principle

The experiment has four main stages:

Stage	Description
1	Reverse transcription of the TNF-α RNA.
2	Amplification of the cDNA, monitored online with the SYBR Green I dye.
3	Evaluation of the fluorescence signals with the **Quantification** program.
4	Analysis of the melting data with the **Melting Curve** program.

Sample Material

Stock solutions (1×10^6, 1×10^5, 1×10^4, and 1×10^3 copies/µl) of *in vitro* transcribed TNF-α RNA, diluted in a solution containing 10 ng/µl MS2 RNA.

Assay Time

The total assay time is approx. 1 hour. The assay time includes the following:

Step	Time
RT-PCR setup	15 min
Reverse transcription	10 min
Amplification	30 min
Evaluation of results	5 min

Additional Material Required

To perform this assay you will need the following:

- LightCycler - RNA Amplification Kit SYBR Green I (Cat. No. 2 015 137)
- LightCycler - Control Kit RNA (Cat. No. 2 015 153)
- Standard benchtop microcentrifuge with a rotor for 2.0 ml tubes
  Note: Centrifuge adapters supplied with the LightCycler fit into a standard microcentrifuge rotor with 2 ml reaction tubes.

3.2.2 Programming the Experimental Protocol

Overview

The experimental protocol contains four cycle programs:

- Program 1: Reverse transcription of the template RNA
- Program 2: Denaturation of cDNA/RNA hybrid
- Program 3: Amplification of cDNA
- Program 4: Melting curve analysis (for product identification)
- Program 5: Cooling of the rotor and thermal chamber

Note: Directly after setting up the last program, you must also set the fluorescence parameters (as described later in this section).

Program 1: Reverse Transcription

Set up the program for reverse transcription as follows:

Step	Action
1	From the **Front Screen**, click on the **Run** button to access the **Programming Screen**.
2	• Create a new **Experimental Protocol** by clicking on the **New Experiment** button in the **Experiment/RUN/Exit window** (upper left portion of screen). ***Result:*** The computer will now ask for name and path of the experimental protocol to be programmed. • Type a file name for the protocol and specify the file path. • Click **OK**.
3	In the **Cycle Program Field** (below the experiment file name in the upper right corner of the screen), click on **Add,** type the name of the program, and click **OK.**
4	In the **Cycle Program Data** and **Temperature Targets** windows (center of screen), enter the following program parameters:

Parameter	Value
Cycles	1
Analysis Mode	None
Temperature Targets	**Segment 1** **Note:** *Click on **INS** to insert each new segment!*
Target Temperature (°C)	55
Incubation time (h:min:s)	10:00
Temperature Transition Rate (°C/s)	20.0
Secondary Target Temperature (°C)	0
Step Size (°C)	0
Step Delay (cycles)	0
Acquisition Mode	None

Result: The program is saved automatically.

Continued on next page

3.2.2 Programming the Experimental Protocol, Continued

Program 2: Denaturation

To inactivate the reverse transcriptase and to denature the cDNA, program a short denaturation step, as follows:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>• To add a new program, click on the last program in the Cycle Program list, then click on Add.
• Type the name of the program, then click OK.</td></tr>
<tr><td>2</td><td>In the Cycle Program Data and Temperature Targets windows, enter the following program parameters:

<table>
<tr><th>Parameter</th><th>Value</th></tr>
<tr><td>Cycles</td><td>1</td></tr>
<tr><td>Analysis Mode</td><td>None</td></tr>
<tr><td>Temperature Targets</td><td>Segment 1
<u>Note:</u> Click on INS to insert each new segment!</td></tr>
<tr><td>Target Temperature (°C)</td><td>95</td></tr>
<tr><td>Incubation time (h:min:s)</td><td>30</td></tr>
<tr><td>Temperature Transition Rate (°C/s)</td><td>20</td></tr>
<tr><td>Secondary Target Temperature (°C)</td><td>0</td></tr>
<tr><td>Step Size (°C)</td><td>0</td></tr>
<tr><td>Step Delay (cycles)</td><td>0</td></tr>
<tr><td>Acquisition Mode</td><td>None</td></tr>
</table>

<u>Result:</u> The program is saved automatically.</td></tr>
</table>

Continued on next page

Program 3: Amplification

During amplification, the fluorescent signal is measured (acquired) at the end of the elongation step. Set up the cycling program as follows:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>• To add a new program, click on the last program in the Cycle Program list, then click on Add.
• Type the name of the program, then click OK.</td></tr>
<tr><td>2</td><td>In the Cycle Program Data and Temperature Targets windows, enter the following program parameters:
<table>
<tr><th>Parameter</th><th colspan="3">Value</th></tr>
<tr><td>Cycles</td><td colspan="3">45</td></tr>
<tr><td>Analysis Mode</td><td colspan="3">Quantification</td></tr>
<tr><td>Temperature Targets</td><td>Segment 1</td><td>Segment 2</td><td>Segment 3
<u>Note:</u> Click on INS to insert each new segment!</td></tr>
<tr><td>Target Temperature (°C)</td><td>95</td><td>55</td><td>72</td></tr>
<tr><td>Incubation time (h:min:s)</td><td>1</td><td>10</td><td>13</td></tr>
<tr><td>Temperature Transition Rate (°C/s)</td><td>20.0</td><td>20.0</td><td>2.0</td></tr>
<tr><td>Secondary Target Temperature (°C)</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Step Size (°C)</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Step Delay (cycles)</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Acquisition Mode</td><td>None</td><td>None</td><td>Single</td></tr>
</table>
<u>Result:</u> The program is saved automatically.</td></tr>
</table>

Continued on next page

3.2.2 Programming the Experimental Protocol, Continued

Program 4: Melting Curve Analysis

Set up the program for the melting curve analysis (which will be performed immediately after completion of amplification) as follows:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>• To add a new program, click on the last program in the Cycle Program list, then click on Add.
• Type the name of the program, then click OK.</td></tr>
<tr><td>2</td><td>In the Cycle Program Data and Temperature Targets windows, enter the following program parameters:
<table>
<tr><th>Parameter</th><th colspan="3">Value</th></tr>
<tr><td>Cycles</td><td colspan="3">1</td></tr>
<tr><td>Analysis Mode</td><td colspan="3">Melting Curves</td></tr>
<tr><td>Temperature Targets</td><td>Segment 1</td><td>Segment 2</td><td>Segment 3
<u>Note:</u> Click on INS to insert each new segment!</td></tr>
<tr><td>Target Temperature (°C)</td><td>95</td><td>65</td><td>72</td></tr>
<tr><td>Incubation time (h:min:s)</td><td>0</td><td>20</td><td>0</td></tr>
<tr><td>Temperature Transition Rate (°C/s)</td><td>20</td><td>20</td><td>0.2</td></tr>
<tr><td>Secondary Target Temperature (°C)</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Step Size (°C)</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Step Delay (cycles)</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Acquisition Mode</td><td>None</td><td>None</td><td>Cont.</td></tr>
</table>
<u>Result:</u> The program is saved automatically.</td></tr>
</table>

Continued on next page

3.2.2 Programming the Experimental Protocol, Continued

Program 5: Cooling

Before removing the rotor from the instrument, you should cool the rotor and capillaries. Set up the program for the cooling step as follows:

Step	Action
1	• To add a new program, click on the last program in the **Cycle Program** list, then click on **Add**. • Type the name of the program, then click **OK**.
2	In the **Cycle Program Data** and **Temperature Targets** windows, enter the following program parameters: **Parameter** / **Value** Cycles: 1 Analysis Mode: None **Temperature Targets**: **Segment 1** — **Note:** *Click on **INS** to insert each new segment!* Target Temperature (°C): 40 Incubation time (h:min:s): 60 Temperature Transition Rate (°C/s): 20.0 Secondary Target Temperature (°C): 0 Step Size (°C): 0 Step Delay (cycles): 0 Acquisition Mode: None ***Result:*** The program is saved automatically.
3	Save the whole experimental protocol by clicking on the **Save Experiment File** button in the **Experiment/RUN/Exit** window. If you have not already done so, enter a file name.

Setting the Fluorescence Parameters

In the **Display Mode/Fluorimeter Gains** window (upper middle part of screen), set the fluorescence parameters as follows:

- Display Mode: Choose fluorescence channel 1 (**F** 1)
- Set the fluorescence gains as follows:

Fluorimeter Gain	Value
Channel 1 (**F** 1)	5
Channel 2 (**F** 2)	10
Channel 3 (**F** 3)	10

Continued on next page

3.2.2 Programming the Experimental Protocol, Continued

Naming the Samples

After programming the temperature profiles, type in the names and descriptions for the samples as follows:

Step	Action
1	• Click on the **Edit Samples** button in the **Experiment/RUN/Exit** window (left portion of screen). ***Result:*** The **Loading Screen** will appear and a dialog box will ask you to name the RT-PCR run. • Enter a file name and click the **OK** button.
2	The **Loading Screen** contains a **Sample Data** table. In this table, enter the appropriate information for all the samples: • Overwrite the standard settings in the **Sample Name** column (Sample 1, Sample 2, ...) with your sample names. ***Note:*** You can also add the sample names during a PCR run! Refer to Chapter B for more detailed information. • Specify the type of sample (e.g., standard, negative control, unknowns, etc.) in the **Type** column. • In the remaining columns of the table, list all additional sample features [replicates, concentrations (if known), and descriptive notes] ***Note:*** For standards that have known template concentrations, you may note those concentrations in the "Concentration" column of the table. This column of the table can only be filled before the run is started! ***Caution:*** Do not designate a negative ("no template") control as a known standard with "**0"** concentration. • Enter the number of samples in the **Number of Samples** field (lower left corner of window) and click **OK**.
3	In the lower left corner of the screen, set the temperature to 55°C. ***Note:*** This is the temperature the instrument will reach during the "seek process". It is the correct temperature for the reverse transcription step.
4	Click the **Done** button to return to the **Programming Screen.**

3.2.3 Preparing RT-PCR Samples and Starting the Program

Introduction

Before running the LightCycler instrument, prepare the samples. Preparation involves four major stages:

Stage	Description
1	Thawing the required solutions.
2	Preparing the sample mixes.
3	Loading the capillaries and placing them into the sample carousel.
4	Placing the sample carousel into the thermal chamber and starting the program.

Thawing the Solutions

Before starting the assay, thaw all the following reagents and store them on ice:

From the ...	Thaw the...
LightCycler - RNA Amplification Kit SYBR Green I	• LightCycler - RT-PCR Enzyme Mix (vial 1, green cap) • LightCycler - RT-PCR Reaction Mix SYBR Green I (vial 2, green cap) • $MgCl_2$ stock solution (vial 3, blue cap)
LightCycler - Control Kit RNA	• Cytokine RNA (vials 1–4, purple caps) • Cytokine Primer mix (vial 5, white cap) • H_2O, sterile, PCR grade (vial 7, colorless cap)

Setting Up Capillaries

Place LightCycler capillaries into precooled centrifuge adapters. Use one capillary for every sample to be analyzed.
Note: Do not touch the surface of the capillaries with bare fingers. Always wear gloves when handling the capillaries.

Continued on next page

3.2.3 Preparing RT-PCR Samples and Starting the Program,
Continued

Negative Control

To prepare a negative control, replace the template DNA with PCR grade water (vial 7, colorless cap) in a reaction mix containing all other reaction components.
Note: Always run a negative control with the samples!

Preparation of Sample Mixes

Prepare RT-PCR samples as follows:
Note: This procedure is designed for a 20 µl standard reaction.

Step	Action
1	In a 1.5 ml reaction tube on ice, prepare enough master mix for all samples to be analyzed. Add the components in the order given below. ***Note:*** The table below lists the amounts of each component needed for one reaction. To determine the total volume of each component needed, multiply the amount in the "Volume" column by (n+1), where n = the number of reactions to be cycled.
2	• Pipet 19 µl master mix into each precooled capillary. • Add 1 µl of each cytokine RNA template (vials 1-4).

Reagent	Volume per reaction	Final conc.
H_2O, sterile, PCR grade (vial 7, colorless cap)	13 µl	
LightCycler - RT-PCR Reaction Mix SYBR Green I (vial 2, green cap)	2 µl	1x
$MgCl_2$, stock solution (vial 3, blue cap)	1.6 µl	6 mM
Cytokine Primer Mix (vial 5, white cap)	2.0 µl	0.5 µM each
LightCycler - RT-PCR Enzyme Mix (vial 1, red cap)	0.4 µl	1x
Final volume	**19 µl**	

Continued on next page

3.2.3 Preparing RT-PCR Samples and Starting the Program,
Continued

Preparation of Sample Mixes (continued)

Step	Action
3	• Seal each capillary with a stopper and place the adapter, containing the capillary, into a standard benchtop microcentrifuge. • Centrifuge at 700 x g (3000 rpm in a standard benchtop microcentrifuge) for 5 s. ***Note:*** Arrange the centrifuge adapters so the rotor is in balance, i.e., opposing samples should weigh the same.
4	Place the capillaries in the rotor of the LightCycler. ***Note:*** Samples can be also kept in the cooling block at 4°C until they are placed into the sample carousel.

Program Start

Start the run as follows:

Step	Action
1	Place the loaded sample carousel into the thermal chamber.
2	Click the **Run** button. ***Result:*** During the "Seek Process", the LightCycler will find the optimal capillary position for fluorescence detection of each individual sample and then start the programmed run.

End of Experimental Protocol

When the protocol is finished, the LightCycler will automatically exit the **Running Screen** and display the collected data on the **LightCycler Data Analysis (LCDA) Front Screen**.

3.2.4 Data Analysis with the Quantification Program

Starting Quantification Analysis

To start data evaluation with the **Quantification Program**, use this procedure:

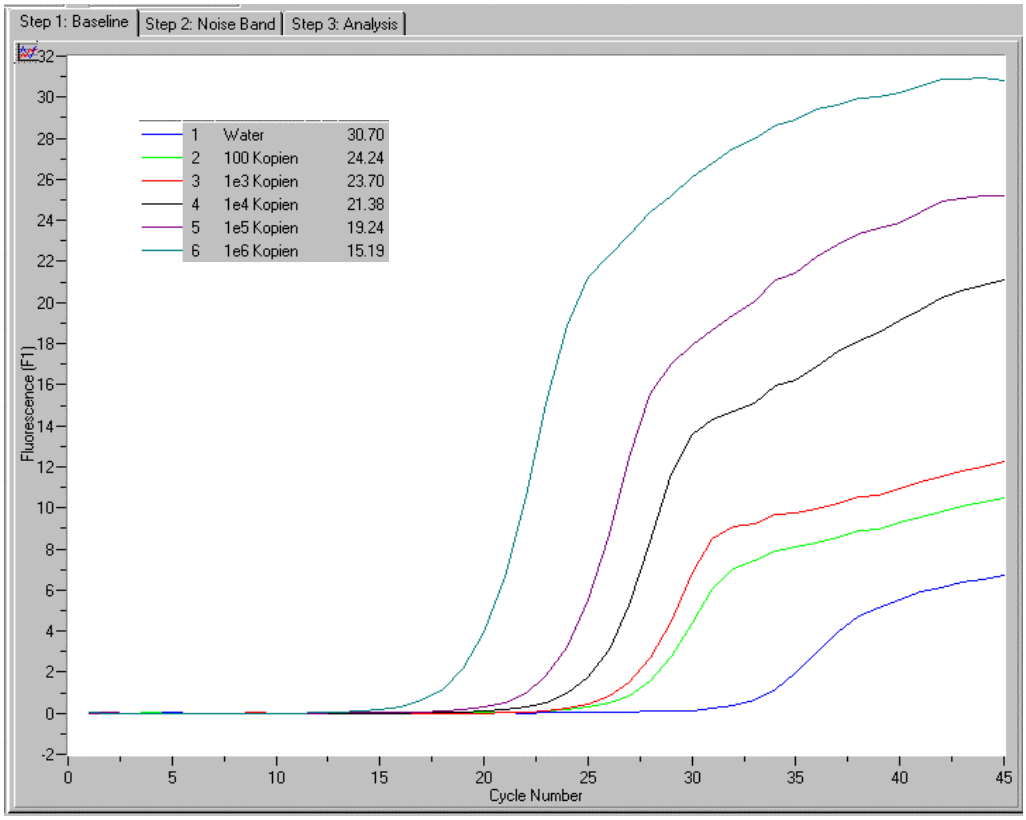
Step	Action
1	Do one of the following: • If the instrument has just finished a run and the **LCDA Front Screen** is displayed, skip Steps 2 and 3 below and go to Step 4. • If no run has been performed, (e.g. if you want to analyze data collected during a previous run), go to Step 2 below.
2	• Click on the **Data Analysis button** on the **LightCycler Front Screen**. • In the window that appears, select "Open" from the **File** menu. ***Result:*** The **Select Data File** window will open.
3	Open the appropriate run file by highlighting a file name and clicking on **Open**. ***Result:*** The **LCDA Front Screen** will appear.
4	Make sure that the amplification segment of the upper temperature chart is selected for analysis. ***Note:*** To select the amplification segment, choose the appropriate (amplification) program segment of the protocol in the **Select a Program** pull down menu.
5	Verify that the fluorescence display mode for the lower graph is **F1** (in the window to the left of the graph).
6	Enter the **Quantification Screen** by clicking on the **Quantification** button (top center portion of screen).
7	Optimize the data display and analyze the data as described in Section 5 of Chapter B. ***Note:*** See Section 4 of this chapter for more information on the quantification process.

Continued on next page

3.2.4 Data Analysis with the Quantification Program, Continued

Typical result

The picture below shows quantification data from a typical run of the procedure described in Section 3.2.



Result: In the LightCycler SYBR Green I procedure, as few as 100 copies (green line) of template RNA produces fluorescent signals significantly higher than that of the negative control (water).

3.2.5 Data Analysis with the Melting Curve Program

Overview

The **Melting Curve** program can be used for additional analysis of the experimental data. Because primer-dimers melt at much lower temperatures than specific PCR products, this program can differentiate signals obtained from the specific PCR product from those of primer-dimers.

Selecting the Program

For data evaluation with the **Melting Curve** program, do the following:

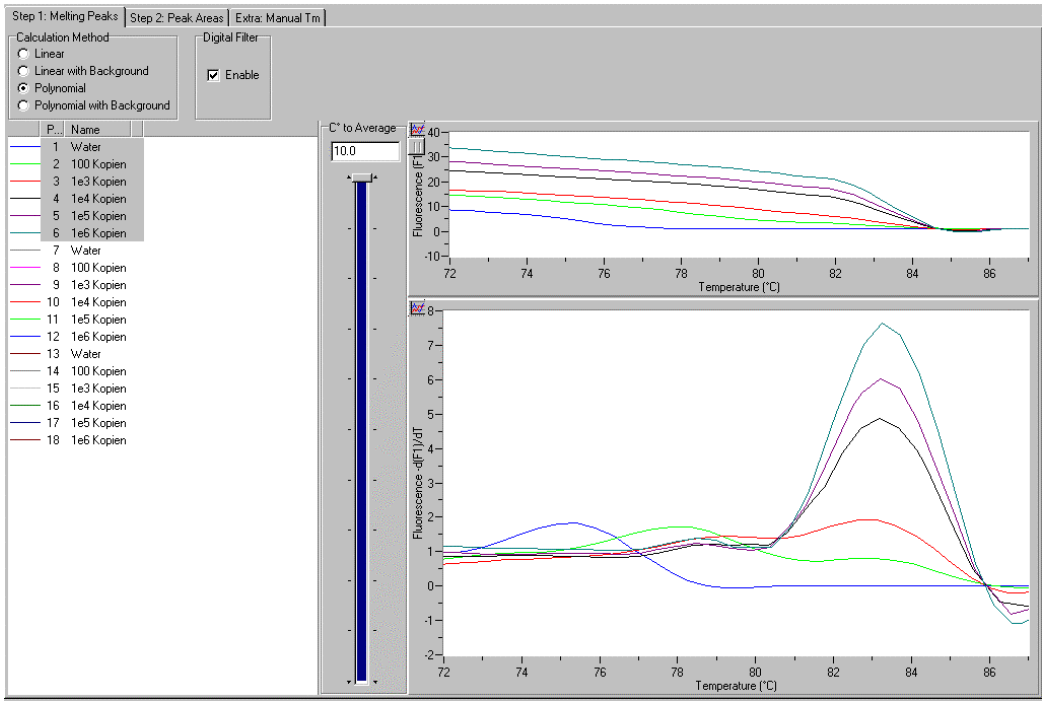
Step	Action
1	Return to the **LCDA Front Screen** by minimizing or closing the open window.
2	Choose the Melting Curve program segment of the protocol by clicking on the **Select a Program** button and highlighting the appropriate program segment. ***Result:*** The cursors in the Temp Chart will automatically be set to the beginning and end of the Melting Curve.
3	Click on the **Melting Curve** button (top center portion of screen) to enter the **Melting Curve Analysis Screen**.
4	Verify that fluorescence is set to **F 1** for the y-axis of the lower graph (lower left corner of graph).
5	Optimize the data display and analyze the melting curve as described in Section 6 of Chapter B.

Continued on next page

3.2.5 Data Analysis with the Melting Curve Program, Continued

Typical Result

The picture below shows melting curve data from a typical run of the procedure described in Section 3.2.



Result: The Melting Curve Program can discriminate between primer-dimers and specific cytokine product (which melts at a higher temperature than the primer-dimers). At higher template concentrations, most amplicons are from the target sequence; few primer-dimers are amplified. At lower template concentrations, significant primer-dimer amplification occurs, but the program can still identify a fluorescent product-specific signal. In the negative control sample, only primer dimers are amplified.

3.3 One-Step RT-PCR of TNF-α RNA with Hybridization Probes

3.3.1 Test Overview

Introduction

The procedure below describes reverse transcription and subsequent amplification of Tumor Necrosis Factor-α (TNF-α) RNA in a single tube. The reaction is performed in the presence of TNF-α specific hybridization probes, which allow quantification of the reaction product.

Test Principle

The procedure has three main stages:

Stage	Description
1	Reverse transcription of the TNF-α mRNA.
2	Amplification of the cDNA, monitored online with hybridization probes.
3	Evaluation of the results with the **Quantification** program.

Sample Material

Stock solutions (1×10^6, 1×10^5, 1×10^4, and 1×10^3 copies/µl) of *in vitro* transcribed TNF-α RNA, diluted in a solution containing 10 ng/µl MS2 RNA.

Assay Time

The total assay time is approx. 1 hour. The assay includes the following:

Step	Time
Preparation of PCR samples	15 min
Reverse transcription	10 min
Amplification	30 min
Evaluation of results	5 min

Additional Material Required

To perform this assay you will need the following:

- LightCycler - RNA Amplification Kit Hybridization Probes (Cat. No. 2 015 145)
- LightCycler - Control Kit RNA (Cat. No. 2 015 153)
- Standard benchtop microcentrifuge with a rotor for 2.0 ml tubes
  Note: Centrifuge adapters supplied with the LightCycler fit into a standard microcentrifuge rotor with 2 ml reaction tubes.

3.3.2 Programming the Experimental Protocol

Programming

The Experimental Protocol contains four cycle programs:

- Program 1: Reverse transcription of the template RNA
- Program 2: Denaturation of cDNA/RNA hybrid
- Program 3: Amplification of cDNA
- Program 4: Cooling the rotor and the thermal chamber

Note: Directly after setting up the last program, you must also set the fluorescence parameters (as described later in this section).

Program 1: Reverse Transcription

Set up the program for reverse transcription as follows:

Step	Action
1	From the **Front Screen**, click on the **Run** button to access the **Programming Screen**.
2	• Create a new **Experimental Protocol** by clicking on the **New Experiment** button in the **Experiment/RUN/Exit window** (upper left portion of screen). ***Result:*** The computer will now ask for name and path of the experimental protocol to be programmed. • Type a file name for the protocol and specify the file path. • Click **OK**.
3	In the **Cycle Program** list (below the experiment file name in the upper right corner of the screen) click on **Add,** type the name of the program and click **OK.**
4	In the **Cycle Program Data** and **Temperature Targets** windows (center of screen), enter the following program parameters:

Parameter	Value
Cycles	1
Analysis Mode	None
Temperature Targets	**Segment 1** **Note:** *Click on **INS** to insert each new segment!*
Target Temperature (°C)	55
Incubation time (h:min:s)	10:00
Temperature Transition Rate (°C/s)	20.0
Secondary Target Temperature (°C)	0
Step Size (°C)	0
Step Delay (cycles)	0
Acquisition Mode	None

Result: The program is saved automatically.

Continued on next page

Program 2: Denaturation

To inactivate the reverse transcriptase and to denature the cDNA, program a short denaturation step as follows:

Step	Action
1	• To add a new program, click on the last program in the **Cycle Program** list, then click on **Add**. • Type the name of the program, then click **OK**.
2	In the **Cycle Program Data** and **Temperature Targets** windows, enter the following program parameters:

Parameter	**Value**
Cycles	1
Analysis Mode	None
Temperature Targets	**Segment 1** **Note:** *Click on **INS** to insert each new segment!*
Target Temperature (°C)	95
Incubation time (h:min:s)	30
Temperature Transition Rate (°C/s)	20.0
Secondary Target Temperature (°C)	0
Step Size (°C)	0
Step Delay (cycles)	0
Acquisition Mode	None

Result: The program is saved automatically.

Continued on next page

Program 3: Amplification

During amplification, the fluorescent signal is measured (acquired) at the end of the annealing step. Set up the cycling program as follows:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>• To add a new program, click on the last program in the Cycle Program list, then click on Add.
• Type the name of the program, then click OK.</td></tr>
<tr><td>2</td><td>In the Cycle Program Data and Temperature Targets windows, enter the following program parameters:
<table>
<tr><th>Parameter</th><th colspan="3">Value</th></tr>
<tr><td>Cycles</td><td colspan="3">45</td></tr>
<tr><td>Analysis Mode</td><td colspan="3">Quantification</td></tr>
<tr><td>Temperature Targets</td><td>Segment 1</td><td>Segment 2</td><td>Segment 3
<u>Note:</u> Click on INS to insert each new segment!</td></tr>
<tr><td>Target Temperature (°C)</td><td>95</td><td>50</td><td>72</td></tr>
<tr><td>Incubation time (h:min:s)</td><td>1</td><td>15</td><td>13</td></tr>
<tr><td>Temperature Transition Rate (°C/s)</td><td>20.0</td><td>20.0</td><td>2.0</td></tr>
<tr><td>Secondary Target Temperature (°C)</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Step Size (°C)</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Step Delay (cycles)</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Acquisition Mode</td><td>None</td><td>Single</td><td>None</td></tr>
</table>
<u>Result:</u> The program is saved automatically.</td></tr>
</table>

Continued on next page

3.3.2 Programming the Experimental Protocol, Continued

Program 4: Cooling

Before removing the rotor from the instrument, you should cool the rotor and capillaries. Set the values for the cooling step as follows:

Step	Action
1	• To add a new program, click on the last program in the **Cycle Program** list, then click on **Add**. • Type the name of the program, then click **OK**.
2	In the **Cycle Program Data** and **Temperature Targets** windows, enter the following program parameters: **Parameter** / **Value** Cycles: 1 Analysis Mode: None **Temperature Targets**: **Segment 1** **Note:** *Click on **INS** to insert a new segment!* Target Temperature (°C): 40 Incubation time (h:min:s): 60 Temperature Transition Rate (°C/s): 20.0 Secondary Target Temperature (°C): 0 Step Size (°C): 0 Step Delay (cycles): 0 Acquisition Mode: None ***Result:*** The program is saved automatically.
3	Save the whole experimental protocol by clicking on the **Save Experiment File** button in the **Experiment/RUN/Exit** window. If you have not already done so, enter a file name.

Setting the Fluorescence Parameters

In the **Display Mode/Fluorimeter Gains** window (upper middle part of screen), set the fluorescence parameters as follows:

- Display Mode: Choose fluorescence channel 2 (**F** 2)
- Set the fluorescence gains as follows:

Fluorimeter Gain	Value
Channel 1 (**F** 1)	1
Channel 2 (**F** 2)	10
Channel 3 (**F** 3)	10

Continued on next page

3.3.2 Programming the Experimental Protocol, Continued

Naming the Samples

After programming the temperature profiles, type in the sample names and features as follows:

Step	Action
1	Click on the **Edit Samples** button in the **Experiment/RUN/Exit** window (left portion of screen). The **Loading Screen** will appear.
2	The **Loading Screen** contains a **Sample Data** table. In this table, enter the appropriate information for all the samples: • Overwrite the standard settings in the **Sample Name** column (Sample 1, Sample 2, ...) with your sample names. ***Note:*** You can also add the sample names during a PCR run! Refer to Chapter B for more detailed information. • Specify the type of sample (e.g., standard, negative control, unknowns, etc.) in the **Type** column. • In the remaining columns of the table, list all additional sample features [replicates, concentrations (if known), and descriptive notes] ***Note:*** For standards that have known template concentrations, you may note those concentrations in the "Concentration" column of the table. This column of the table can only be filled before the run is started! ***Caution:*** Do not designate a negative ("no template") control as a known standard with "**0"** concentration. • Enter the number of samples in the **Number of Samples** field (lower left corner of window) and click **OK**.
3	In the lower left corner of the screen, set the temperature to 55°C. ***Note:*** This is the temperature the instrument will reach during the "seek process" (initiated after the run has started). It is the correct temperature for the reverse transcription step.
4	Click the **Done** button to return to the **Programming Screen.**

3.3.3 Preparing RT-PCR Samples and Starting the Program

Introduction

Before running the LightCycler instrument, prepare the samples. Preparation involves four major stages:

Stage	Description
1	Thawing the required solutions.
2	Preparing the sample mixes.
3	Loading the capillaries and placing them into the sample carousel.
4	Placing the sample carousel into the thermal chamber and starting the program.

Thawing the Solutions

Before starting the assay, thaw all the following reagents and store them on ice:

From the ...	Thaw the...
LightCycler - RNA Amplification Kit Hybridization Probes	• LightCycler - RT-PCR Enzyme Mix (vial 1, red cap) • LightCycler - RT-PCR Reaction Mix Hybridization Probes (vial 2, red cap) • $MgCl_2$ stock solution (vial 3, blue cap)
LightCycler - Control Kit RNA	• Cytokine RNA (vials 1-4, purple caps) • Cytokine Primer mix (vial 5, white cap) • Cytokine Hybridization Probe mix (vial 6, yellow cap) • H_2O, sterile, PCR grade (vial 7, colorless cap)

Setting Up Capillaries

Place LightCycler capillaries into precooled centrifuge adapters. Use one capillary for every sample to be analyzed.
Note: Do not touch the surface of the capillaries with bare fingers. Always wear gloves when handling the capillaries.

Continued on next page

3.3.3 Preparing RT-PCR Samples and Starting the Program,
Continued

Negative Control

To prepare a negative control, replace the template DNA with PCR grade water (vial 4, colorless cap) in a reaction mix containing all other reaction components.
Note: Always run a negative control with the samples!

Preparation of the Sample Mixes

Prepare PCR samples as follows:
Note: This procedure is designed for a 20 µl standard reaction.

Step	Action
1	In a 1.5 ml reaction tube on ice, prepare enough master mix for all samples to be analyzed. Add the components in the order given below. ***Note:*** The table below lists the amounts of each component needed for one reaction. To determine the total volume of each component needed, multiply the amount in the "Volume" column by (n+1), where n = the number of reactions to be cycled.

Reagent	Volume per reaction	Final concentration
H_2O, sterile, PCR grade (vial 7, colorless cap)	10.2 µl	
LightCycler - RT-PCR Reaction Mix Hybridization Probes (vial 2, red cap)	2 µl	1x
$MgCl_2$, stock solution (vial 3, blue cap)	2.4 µl	6 mM
Cytokine Hybridization Probe mix (vial 6, yellow cap)	2.0 µl	0.2 µM each
Cytokine Primer Mix (vial 5, white cap)	2.0 µl	0.5 µM each
LightCycler - RT-PCR Enzyme Mix (vial 1, red cap)	0.4 µl	
Final volume	**19 µl**	

2	• Pipet 19 µl master mix to each precooled capillary. • Add 1 µl of each cytokine RNA template (vials 1-4).

Continued on next page

3.3.3 Preparing RT-PCR Samples and Starting the Program,
Continued

Preparation of the Sample Mixes (continued)

Step	Action
3	• Seal each capillary with a stopper and place the adapter, containing the capillary, into a standard benchtop microcentrifuge. • Centrifuge at 700 x g (3000 rpm in a standard benchtop microcentrifuge) for 5 s. ***Note:*** Arrange the centrifuge adapters, so the rotor is in balance, i.e., opposing samples should weigh the same.
4	Place the capillaries in the rotor of the LightCycler. ***Note:*** Samples can be also kept in the cooling block at 4°C until they are placed into the sample carousel.

DNA Contamination Control

To test the RNA preparation for DNA contamination, prepare a sample containing template RNA and 2 µl of the LightCycler - DNA Master Hybridization Probes (Cat. No. 2 015 102) (in place of the LightCycler - RT-PCR Reaction Mix Hybridization Probes and the LightCycler - RT-PCR Enzyme Mix).

Program Start

Start the run as follows:

Step	Action
1	Place the loaded sample carousel into the thermal chamber and close the lid.
2	Click the **Run** button. ***Result:*** During the "Seek Process", the LightCycler will find the optimal capillary position for fluorescence detection of each individual sample and then start the programmed run.

End of Experimental Protocol

When the protocol is finished, the LightCycler Software will automatically exit the **Running Screen** and display the collected data on the **LightCycler Data Analysis (LCDA) Front Screen**.

3.3.4 Data Analysis with the Quantification Program

Starting Quantification Analysis

To start data evaluation with the **Quantification Program**, use this procedure:

Step	Action
1	Do one of the following: • If the instrument has just finished a run and the **LCDA Front Screen** is displayed, skip Steps 2 and 3 below and go to Step 4. • If no run has been performed, (e.g. if you want to analyze data collected during a previous run), go to Step 2 below.
2	• Click on the **Data Analysis button** on the **LightCycler Front Screen**. • In the window that appears, select "Open" from the **File** menu. ***Result:*** The **Select Data File** window will open.
3	Open the appropriate run file by highlighting a file name and clicking on **Open**. ***Result:*** The **LCDA Front Screen** will appear.
4	Make sure that the amplification segment of the upper temperature chart is selected for analysis. ***Note:*** To select the amplification segment, choose the appropriate (amplification) program segment of the protocol in the **Select a Program** pull down menu.
5	Verify that the fluorescence display mode for the lower graph is **F2/F1** (in the window to the left of the graph).
6	Enter the **Quantification Screen** by clicking on the **Quantification** button (top center portion of screen).
7	Optimize the data display and analyze the data as described in Section 5 of Chapter B. ***Note:*** See Section 4 of this chapter for more information on the quantification process.

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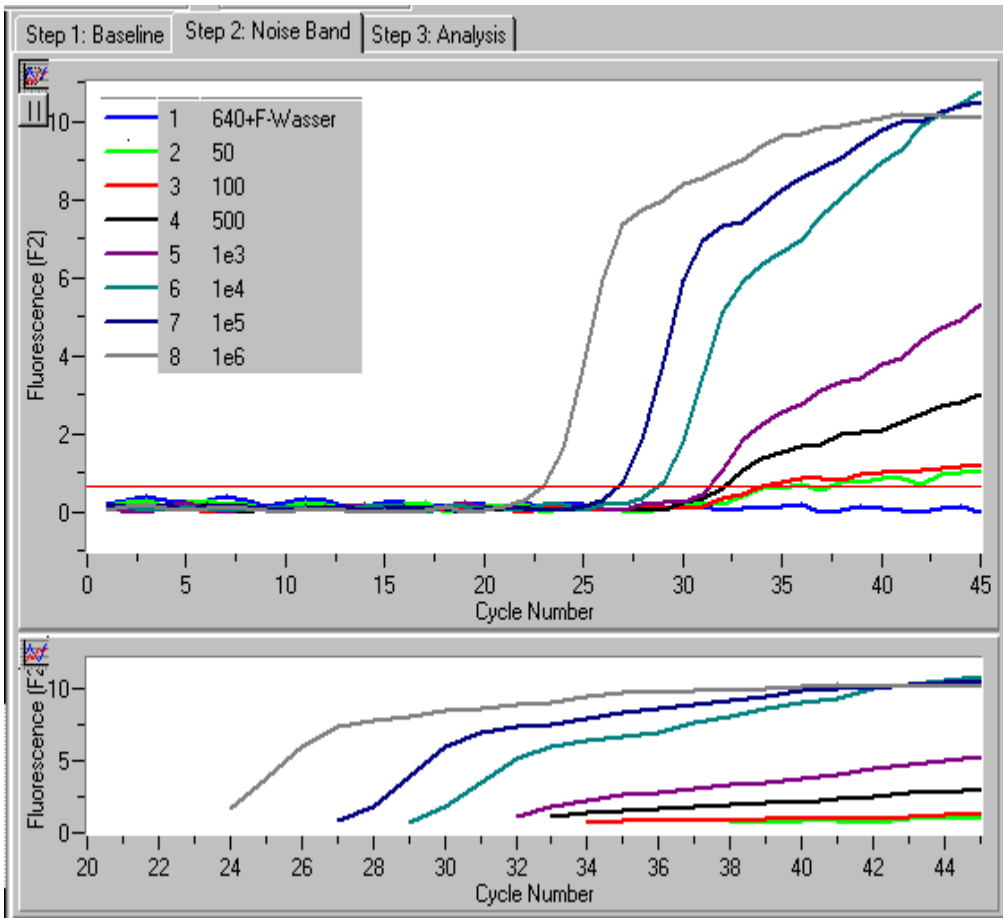
3.3.4 Data Analysis with the Quantification Program, Continued

Typical result

The picture below shows quantification data from a typical run of the procedure described in Section 3.3.



Result: Samples containing as few as 50 copies of template mRNA produce fluorescent signals significantly higher than the negative control.

3.4 Two-Step RT-PCR with SYBR Green I

3.4.1 Test Overview

Introduction

The procedure below describes a Two-Step RT-PCR, in which the reverse transcription of RNA into cDNA is performed outside the LightCycler. The amplification step, with online monitoring, is performed in the LightCycler according to the standard PCR procedure. The cDNA produced in the first step serves as starting sample for the second step. RT-PCR results are interpreted directly following completion of the PCR.

Note: This two-step approach generates a pool of cDNAs, which represent transcripts from all the currently active genes in the cell. Any number of these cDNAs may be amplified from this single sample. Thus, one sample may be used to monitor many genes.

Test Principle

The Two-Step RT-PCR experiment has five main stages:

Stage	Description
1	Set up RT reaction.
2	Perform RT reaction (outside the LightCycler).
3	Set up the PCR amplification.
4	Perform PCR using the LightCycler Instrument.
5	Interpret results.

Sample Material

RNA or mRNA provided by the user.

Assay Time/ Hands on Time

The total assay time is approximately 115 min. The assay time includes the following:

Step	Time
RT setup	15 min
RT reaction	60 min
PCR setup	15 min
LightCycler PCR run	25 min

Continued on next page

3.4.1 Test Overview, Continued

Additional Material Required

- For the RT reaction, one of the following reagents is required:

Reagent	Cat. No.
First Strand cDNA Synthesis Kit for RT-PCR (AMV)	1 483 188
Expand Reverse Transcriptase *	1 785 826

* Not for sale in the USA.

- Standard benchtop microcentrifuge with a rotor for 2.0 ml tubes
  Note: Centrifuge adapters supplied with the LightCycler fit into a standard microcentrifuge rotor with 2 ml reaction tubes.

3.4.2 Programming the Experimental Protocol for the PCR Step

Overview

For the SYBR Green I detection format, the experimental PCR protocol usually contains four cycle programs:

- Program 1: Denaturation of cDNA
- Program 2: Amplification of the target sequence
- Program 3: Melting curve analysis (for product identification)
- Program 4: Cooling of the rotor and the thermal chamber.

Guidelines for Cycling Conditions

The following table gives an overview of typical cycling parameters for PCR.
Note: For details on programming a LightCycler PCR, see the detailed protocols given elsewhere in this chapter (e.g., in Section 2.1.2)

Parameter	Value
Denaturation temperature	95°C
Denaturation time (s)	0
Annealing temperature (°C)	Primer-dependent
Annealing time (s)	5–15
Elongation temperature (°C)	72°
Elongation time (s)	Time (s) = Length of product [bp] ÷ 25

Setting the Fluorescence Parameters

In the **Display Mode/Fluorimeter Gains** window (upper middle part of screen), set the fluorescence parameters as follows:

- Display Mode: Choose fluorescence channel 1 (**F** 1)
- Set the fluorescence gains as follows:

Fluorimeter Gain	Value
Channel 1 (**F** 1)	5
Channel 2 (**F** 2)	10
Channel 3 (**F** 3)	10

Continued on next page

3.4.2 Programming the Experimental Protocol for the PCR Step,
Continued

Naming the Samples

After programming the temperature profiles, type in the names and descriptions for the samples as follows:

Step	Action
1	Click on the **Edit Samples** button in the **Experiment/RUN/Exit** window (left portion of screen). The **Loading Screen** will appear.
2	The **Loading Screen** contains a **Sample Data** table. In this table, enter the appropriate information for all the samples: • Overwrite the standard settings in the **Sample Name** column (Sample 1, Sample 2, ...) with your sample names. ***Note:*** You can also add the sample names during a PCR run! Refer to Chapter B for more detailed information. • Specify the type of sample (e.g., standard, negative control, unknowns, etc.) in the **Type** column. • In the remaining columns of the table, list all additional sample features [replicates, concentrations (if known), and descriptive notes] ***Note:*** For standards that have known template concentrations, you may note those concentrations in the "Concentration" column of the table. This column of the table can only be filled before the run is started! ***Caution:*** Do not designate a negative ("no template") control as a known standard with "**0"** concentration. • Enter the number of samples in the **Number of Samples** field (lower left corner of window) and click **OK**.
2	Click the **Done** button to return to the **Programming Screen.**

3.4.3 Preparing Samples and Starting the Program

Introduction

Before running the LightCycler instrument, prepare the samples. Preparation involves five major stages:

Stage	Description
1	Performing cDNA synthesis (outside the LightCycler).
2	Thawing the required solutions for the master mix.
3	Preparing the sample mixes.
4	Loading the capillaries and placing them into the sample carousel.
5	Placing the sample carousel into the thermal chamber and starting the program.

Set Up the RT Reaction

Perform cDNA synthesis with either the First Strand cDNA Synthesis Kit for RT-PCR or the Expand Reverse Transcriptase. Use the standard protocol given in the pack insert of the product.

Note: These protocols assume that each standard RT reaction will contain 1 µg total RNA template in a final volume of 20 µl.

Set Up the PCR

Perform amplification and online analysis of the cDNA with the LightCycler Instrument.

Note: The amplification conditions and cycle conditions have to be optimized for each cDNA template. Use the following table for optimization guidelines:

Parameter	Recommendation
$MgCl_2$ concentration	Has to be optimized according to specific requirements of the target cDNA.
Primers	Can be used at the recommended concentration, but should also be tested for optimal performance after $MgCl_2$ titration.
Cycle conditions	Annealing temperature and holding times have to be optimized.

Optional: Dilution Series for the cDNA

For best results:

- Prepare a fresh series of cDNA dilutions.
- Perform the PCR with 2 µl of undiluted cDNA and/or with 2 µl of each cDNA dilution as DNA template.

Caution: Excess cDNA can inhibit PCR. Do not use more than 8 µl of undiluted cDNA as DNA template in a final reaction volume of 20 µl.

Continued on next page

3.4.3 Preparing Samples and Starting the Program, Continued

Negative Control

To prepare a negative control, replace the template DNA with PCR grade water (vial 3, LightCycler DNA Master SYBR Green I) in a reaction mix containing all other reaction components.
Note: Always run a negative control with the samples!

Thawing the Solutions

Thaw the following reagents, mix gently and store on ice:

From...	Thaw the...
LightCycler - DNA Master SYBR Green I	• LightCycler - DNA Master SYBR Green I (vial 1, green cap) • $MgCl_2$ stock solution (vial 2, blue cap) • H_2O sterile, PCR grade (vial 3, colorless cap)
User-provided reagents	• cDNA • Primers

Setting Up Capillaries

Place LightCycler capillaries into precooled centrifuge adapters. Use one capillary for every sample to be analyzed.
Note: Do not touch the surface of the capillaries with bare fingers. Always wear gloves when handling the capillaries.

Continued on next page

3.4.3 Preparing Samples and Starting the Program, Continued

Preparation of Sample Mixes

Prepare PCR samples as follows:
Note: This procedure is designed for a 20 µl standard reaction.

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>In a 1.5 ml reaction tube on ice, prepare enough master mix for all samples to be analyzed. Add the components in the order given below.
Note: The table below lists the amounts of each component needed for one reaction. To determine the total volume of each component needed, multiply the amount in the “Volume” column by (n+1), where n = the number of reactions to be cycled.
<table>
<tr><th>Component</th><th>Volume</th><th>Final conc.</th></tr>
<tr><td>H_2O, sterile, PCR grade (vial 4, colorless cap)</td><td>To 18µl (final volume)</td><td></td></tr>
<tr><td>$MgCl_2$ stock solution (vial 2, blue cap)</td><td>x µl</td><td>1–5 mM*</td></tr>
<tr><td>Primers</td><td>y µl</td><td>0.3-1 µM each*</td></tr>
<tr><td>LightCycler - DNA Master Hybridization Probes (vial 1, red cap)</td><td>2.0 µl</td><td>1x</td></tr>
<tr><td>Total volume</td><td>18 µl</td><td></td></tr>
</table>
* Determined experimentally.</td></tr>
<tr><td>2</td><td>• Pipet 18 µl master mix into each precooled capillary.
• Add 2 µl of the appropriate pre-diluted cDNA.</td></tr>
<tr><td>3</td><td>• Seal each capillary with a stopper and place the adapter, containing the capillary, into a standard benchtop centrifuge.
• Centrifuge at 700 x g (3000 rpm in a standard benchtop centrifuge) for 5 s.
Note: Arrange the centrifuge adapters so the rotor is in balance, i.e., opposing samples should weigh the same.</td></tr>
<tr><td>4</td><td>Place the capillaries in the rotor of the LightCycler.
Note: Samples can be also kept in the cooling block at 4°C until they are placed into the sample carousel.</td></tr>
</table>

Continued on next page

3.4.3 Preparing Samples and Starting the Program, Continued

Program Start

Start the run as follows:

Step	Action
1	Place the loaded sample carousel into the thermal chamber.
2	Click the **Run** button. ***Result:*** During the "Seek Process", the LightCycler will find the optimal capillary position for fluorescence detection of each individual sample and then start the programmed run.

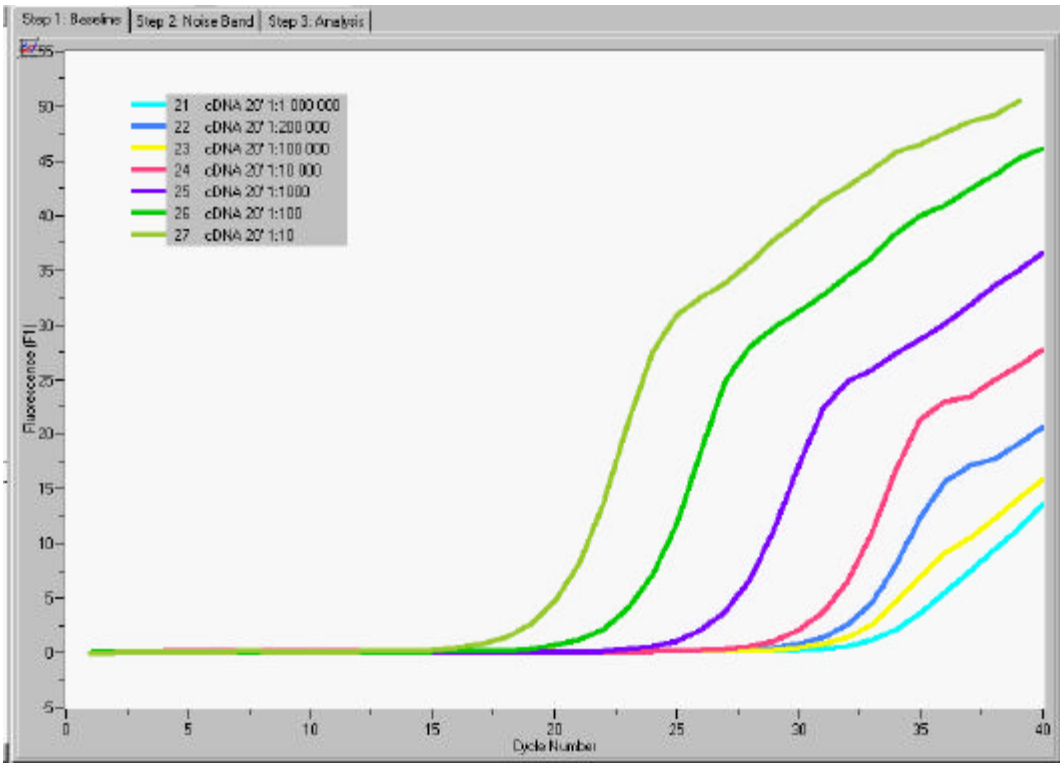
End of Experimental Protocol

When the protocol is finished, the LightCycler Software will automatically exit the **Running Screen** and display the collected data on the **LightCycler Data Analysis (LCDA) Front Screen**.

3.4.4 Typical Results

Typical Results: Quantification

In a Two-Step RT-PCR procedure, 1 µg human total liver RNA was reverse transcribed with AMV reverse transcriptase in a 20 µl total reaction volume. After reverse transcription, 1 µl of the RT reaction mixture, containing the cDNA from 50 ng template RNA, was serially diluted and amplified with the LightCycler - DNA Master SYBR Green I and Cyclophilin A-specific primers. SYBR Green I dye was used to detect the product.
The picture below shows the results from that RT-PCR.



Result: As few as 50 pg RNA, which would be the amount of RNA from 1 cell, gives a measurable fluorescent signal.

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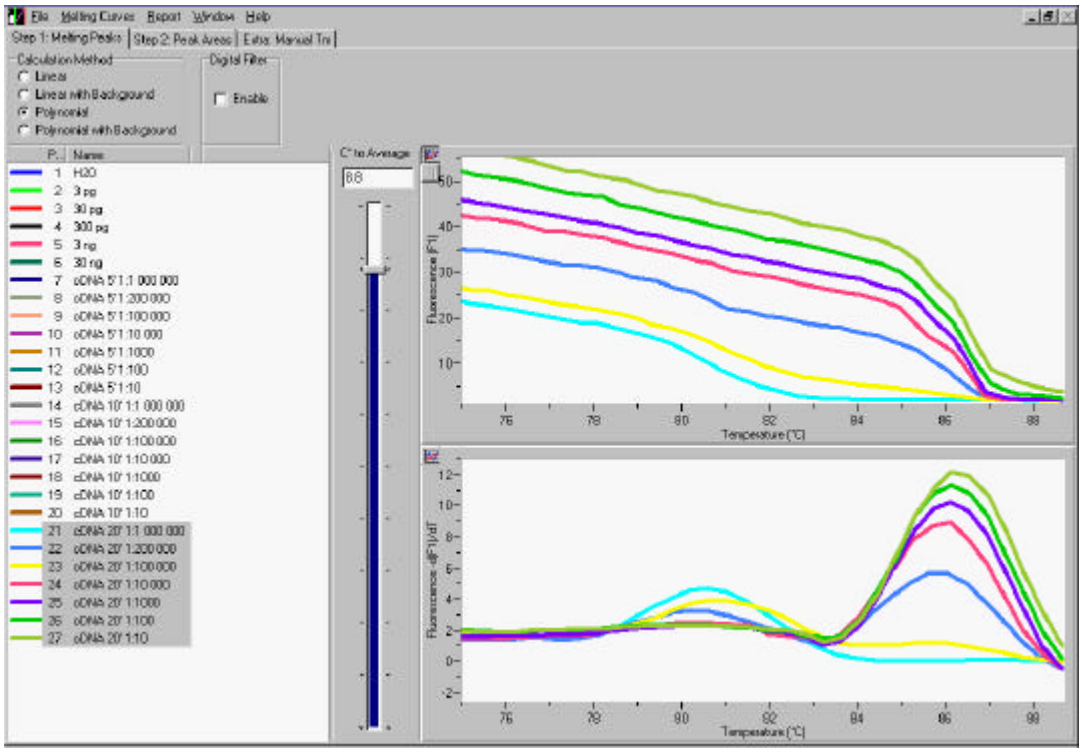
3.4.4 Typical Results, Continued

Typical Results: Melting Curve Analysis

Fluorescence signals caused by primer-dimers may superimpose on the signals obtained from the specific product. However, these two signals can easily be distinguished with a Melting Curve analysis. The picture below shows the results of a Melting Curve analysis on the RT-PCR results from the previous page.



Result: Most cDNA dilutions produce a signal that is easily distinguished from primer-dimer contaminant signals. However, the $1:10^6$ dilution (0.05 pg RNA), the $1:10^5$ dilution (0.5 pg RNA), and the $1:10^4$ dilution (5 pg RNA) do not produce a specific product signal.

How the LightCycler Software Operates

Version 3 of the LightCycler Software provides two different methods for quantification: the Fit Points Method and the Second Derivative Maximum Method. In brief, here is how these two methods work:

- In the Fit Points Method, the user may select the log-linear phase (in which the concentration of the amplified target increases logarithmically), as follows:
  - By setting the Noise Band, the user causes data acquired before the log-linear phase to be discarded.
  - By manually choosing the data points to be evaluated (Fit Points), the user can identify the linear part on the logarithmic plot of the entire run.
  - By setting the Crossing Line, the user can identify the crossing cycle, which is dependent upon template concentration.

  After the user sets the above parameters, the instrument generates a standard curve by plotting the known concentrations of the standard samples versus the crossing points. This curve allows calculation of the template concentration in the unknowns.
- The Second Derivative Maximum Method uses a data driven algorithm to identify the log-linear phase and crossing points automatically. No user input is required for this analysis method. This automatic evaluation method ensures maximum reproducibility from run to run.

Note: For a more detailed description of these methods, see Section B. See Section 4.0.1 of this chapter for guidelines on selecting the most appropriate quantification method for a particular application.

Expected Precision

The percent coefficient of variation (%CV) for quantification analyses performed on the LightCycler have averaged about 25%. This level of precision should allow detection of targets that differ in copy number by a factor 2.

Note: %CV = (standard deviation ÷ the mean) x 100.

Tip: The %CV of a quantification analysis depends upon copy number. If copy number is 10^4–10^6, %CVs can be less than 6%. If copy number is 1–10, the %CV can be as high as 200%. Therefore, putting more copies of your target template into the reaction will increase the precision of your quantification.

4. Quantification with the LightCycler

Limitations of Conventional PCR Methods for Quantification

In performing experiments that explore physiological processes, researchers often need to reliably quantify the copy number of a specific target. In theory, they can quantify nucleic acids by PCR, using conventional methods such as:

- Limiting dilution-PCR,
- Ratio-PCR (also called non-competitive or relative PCR), and
- Competitive PCR.

However, in practice these protocols have serious limitations, including:

- The protocols are tedious.
- None of these protocols can guarantee accurate measurement of a reaction during the log-linear phase. (Yet, ideally, quantitative PCR measurements should be made during the few informative log-linear cycles that occur during a PCR experiment.)
  Note: Competitive PCR permits quantification in the plateau phase. However, during the plateau phase, net synthesis is drastically reduced and differences in initial starting template concentrations may be masked.

Quantification with the LightCycler

The LightCycler can quantify nucleic acids in unknown samples by direct comparison to external standards, which are amplified in parallel reactions. For effective quantification with external standards, the assay must meet the following criteria:

- Quantification occurs only during the log-linear cycles of the reaction, and
- Amplification efficiency of standards and unknowns must be similar.

Fortunately, the real-time monitoring of reactions, which is possible in the LightCycler, simplifies the design of a valid assay that meets these criteria.
Note: External standards are available in a remarkably wide range of concentrations. Therefore, the LightCycler method offers significant advantages over conventional methods for quantification of both low and high concentrations of target.

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4.0.1 Guidelines for Successful Quantification Runs

Quantification with the Two LightCycler Detection Formats

The LightCycler Hybridization Probes format provides sequence-specific detection and ensures that only the intended target is quantified. However, the SYBR Green I format will quantify all amplified DNA, regardless of sequence. Therefore, it may be necessary to compensate for signals caused by primer-dimers and non-specific amplicons, since these signals can superimpose on fluorescence signals from the amplicon of interest (specific PCR product). To eliminate this interference, choose one of these approaches:

- Either acquire (measure) fluorescence at a temperature above the melting point of the primer-dimers (which will be approx. 10°C lower than the melting point of most specific PCR products), or
- Use anti-Taq antibodies to perform a Hot Start PCR (see Section 2.3.6 of Chapter C).

Choice of Calculation Method

For best results, choose a calculation method for Quantification according to the following guidelines:

Use this method	When...
Second Derivative Maximum Method	• You want a "quick look" at results with a data-driven algorithm. • Starting copy number above 1000 copies. ***Caution:*** Do not choose this method if the emitted fluorescence from the sample is near the lower detection limit. ***Note:*** The second derivative method is not affected by background corrections. Setting the background corrections will only affect the curve display but not the calculation!
Fit Points Method	• You want to analyze a wide range of data. ***Note:*** This method is appropriate for analysis of data from all applications. • Samples contain a wide range of starting copy numbers. • Starting copy number below 1000 copies per tube. ***Note:*** This method also works well when starting copy number is high. • You need backward compatibility with old software.

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Requirements for External Standards

For quantification with external standards to be successful, the target must be amplified with the same efficiency as the standards. To verify this in your system, either examine the slope of the log-linear region during amplification or examine the cycle shift that occurs when the standards and template are diluted.

Note: Tube-to-tube variations in standard samples can affect the accuracy of the standard curve. Reduce this variability by using more points in your standard curve. In general, use at least 3 standards. Also, when preparing a dilution series of the standards, use serial dilution steps that are no more than one order of magnitude apart (e.g., 1:10, 1:100, 1:1000, ...).

Guidelines for Preparing External Standards

Always prepare your standards carefully, since the accuracy of your quantification can be no better than the accuracy of your standards. When preparing standards, please follow the guidelines given in this table:

Requirement	Recommendation
Sequence	The standard should be as homologous as possible to the target to ensure similar amplification efficiency.
Source	• Use either plasmid DNA or purified PCR products as standards. • Linearize plasmid DNA. Supercoiled plasmid DNA lowers reproducibility of the reaction. • Synthesize PCR products in the presence of Uracil-dUTP, to permit carry-over contamination by UNG.
Purity	Purify the standards with the High Pure PCR Product Purification Kit to ensure that they are free of nucleotides and primers that can interfere with determination of concentration.
Determination of concentration	Quantify the amount of standard either by measuring absorbance at 260 nm, or by using fluorescent nucleic acid binding dyes.
Working Concentration	Adjust the working concentration of the standards so you have to add 2 µl of each standard to the final PCR reaction mix. This minimizes variations due to pipetting.
Handling	• Use silanized tubes for diluting standards as well as target DNA. • Add tracer DNA (e.g., sonicated, non-homologous λDNA) to prevent absorption of the nucleic acids to the wall of the reaction tube.

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Preparing the Standard Curve

To prepare an accurate standard curve with the **Fit Points** method, follow these recommendations:

- Use 3–5 standards to generate the standard curve.
- Eliminate single points of the standard curve that do not fit.
- Compare the calculated concentration of your standards, which are derived from the standard curve, with the known copy number of your standards.
- Check the reproducibility of your data.
- Adjust the software settings for Quantification analysis according to the guidelines given in Section 5 of Chapter B.

Note: If you use the **Second Derivative Maximum** method, only the number of standards can be manipulated; all other parameters are fixed.

4.1 Quantification of β-Globin Gene Copies with External Standards and Hybridization Probes

4.1.1 Test Overview

Introduction

The procedure below describes how to quantify the number of copies of the human β-globin gene present in human genomic DNA. The determination uses external standards that have been prepared from genomic DNA; these are amplified in parallel with the unknowns. For maximum specificity, the LightCycler Hybridization Probe format has been chosen for analyzing the data.

Test Principle

The procedure has two main stages.

Stage	Description
1	A fragment of the β-globin gene is amplified from human genomic DNA.
2	After amplification, the fluorescence signals are evaluated with the **Quantification** program.

Sample material

Human, genomic DNA (vial 1 of the LightCycler - Control Kit DNA, Cat. No. 2 015 129)

Assay Time

The total assay time is approx. 55 minutes. The assay includes the following:

Step	Time
Preparation of samples	15 min
Amplification and melting	30 min
Interpretation of the results	10 min

Additional Material Required

To perform this assay, you will need the following:

- LightCycler - Control Kit DNA (Cat. No. 2 015 129)
- LightCycler - DNA Master Hybridization Probes (Cat. No. 2 015 102)
- Standard benchtop microcentrifuge with a rotor for 2.0 ml tubes
  Note: Centrifuge adapters supplied with the LightCycler Instrument fit into a standard microcentrifuge rotor which holds 2 ml reaction tubes.

4.1.2 Programming the Experimental Protocol

Overview

The Experimental Protocol contains three cycle programs:

- Program 1: Denaturation of the human genomic DNA
- Program 2: Amplification of the β-globin gene
- Program 3: Cooling of the instrument

Note: Directly after setting up the last program, you must also set the fluorescence parameters (as described later in this section). In addition, you may name the samples and specify the concentration of known samples.

Program 1: Denaturation

Set up the program for the denaturation of the double-stranded DNA as follows:

Step	Action
1	From the **Front Screen**, click on the **Run** button to access the **Programming Screen**.
2	• Create a new **Experimental Protocol** by clicking on the **New Experiment** button in the **Experiment/RUN/Exit window** (upper left portion of screen). ***Result:*** The computer will now ask for name and path of the experimental protocol to be programmed. • Type a file name for the protocol and specify the file path. • Click **OK**.
3	In the **Cycle Program** list (below the experiment file name in the upper right corner of the screen) click on **Add,** type the name of the program and click **OK.**
4	In the **Cycle Program Data** and **Temperature Targets** windows (center of screen), enter the following program parameters:

Parameter	**Value**
Cycles	1
Analysis Mode	None
Temperature Targets	**Segment 1** ***Note:*** *Click on* ***INS*** *to insert each new segment!*
Target Temperature (°C)	95
Incubation time (h:min:s)	30
Temperature Transition Rate (°C/s)	20.0
Secondary Target Temperature (°C)	0
Step Size (°C)	0
Step Delay (cycles)	0
Acquisition Mode	None

Result: The program is saved automatically.

Continued on next page

Program 2: Amplification

During amplification, the fluorescent signal is measured (acquired) at the end of the annealing phase.
Note: At this stage, the hybridization probes have annealed to their target sequence and a fluorescent signal has been generated, but the Taq polymerase has not yet traveled far enough along the DNA strand to displace the probes.

Set up the cycling program as follows:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>• To add a new program, click on the last program in the Cycle Program list, then click on Add.
• Type the name of the program, then click OK.</td></tr>
<tr><td>2</td><td>In the Cycle Program Data and Temperature Targets windows, enter the following program parameters:
<table>
<tr><th>Parameter</th><th colspan="3">Value</th></tr>
<tr><td>Cycles</td><td colspan="3">45</td></tr>
<tr><td>Analysis Mode</td><td colspan="3">Quantification</td></tr>
<tr><td>Temperature Targets</td><td>Segment 1</td><td>Segment 2</td><td>Segment 3
<u>Note:</u> Click on INS to insert each new segment!</td></tr>
<tr><td>Target Temperature (°C)</td><td>95</td><td>55</td><td>72</td></tr>
<tr><td>Incubation time (h:min:s)</td><td>0</td><td>5</td><td>10</td></tr>
<tr><td>Temperature Transition Rate (°C/s)</td><td>20.0</td><td>20.0</td><td>20.0</td></tr>
<tr><td>Secondary Target Temperature (°C)</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Step Size (°C)</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Step Delay (cycles)</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Acquisition Mode</td><td>None</td><td>Single</td><td>None</td></tr>
</table>
<u>Result:</u> The program is saved automatically.</td></tr>
</table>

Continued on next page

Program 3: Cooling

Before removing the rotor from the instrument, you should cool it. Set up the program for the cooling step as follows:

Step	Action
1	• To add a new program, click on the last program in the **Cycle Program** list, then click on **Add**. • Type the name of the program, then click **OK**.
2	In the **Cycle Program Data** and **Temperature Targets** windows, enter the following program parameters: **Parameter** / **Value** Cycles: 1 Analysis Mode: None **Temperature Targets**: **Segment 1** — **Note:** *Click on **INS** to insert each new segment!* Target Temperature (°C): 40 Incubation time (h:min:s): 60 Temperature Transition Rate (°C/s): 20.0 Secondary Target Temperature (°C): 0 Step Size (°C): 0 Step Delay (cycles): 0 Acquisition Mode: None ***Result:*** The program is saved automatically.
3	Save the whole experimental protocol by clicking on the **Save Experiment File** button in the **Experiment/RUN/Exit** window. If you have not already done so, enter a file name.

Setting the Fluorescence Parameters

In the **Display Mode/Fluorimeter Gains** window (upper middle part of screen), set the fluorescence parameters as follows:

- Display Mode: Choose fluorescence channel 2 (**F** 2).
- Set the fluorescence gains as follows:

Fluorimeter Gain	Value
Channel 1 (**F** 1)	1
Channel 2 (**F** 2)	10
Channel 3 (**F** 3)	10

Continued on next page

4.1.2 Programming the Experimental Protocol, Continued

Naming of samples

After programming the temperature profiles, type in the sample names and features as follows:

Step	Action
1	Click on the **Edit Samples** button in the **Experiment/RUN/Exit** window (left portion of screen). The **Loading Screen** will appear.
2	The **Loading Screen** contains a **Sample Data** table. In this table, enter the appropriate information for all the samples: • Overwrite the standard settings in the **Sample Name** column (Sample 1, Sample 2, ...) with your sample names. ***Note:*** You can also add the sample names during a PCR run! Refer to Chapter B for more detailed information. • Specify the type of sample (e.g., standard, negative control, unknowns, etc.) in the **Type** column. • In the remaining columns of the table, list all additional sample features [replicates, concentrations (if known), and descriptive notes]. ***Note:*** For standards that have known template concentrations, you may note those concentrations in the "Concentration" column of the table. This column of the table can only be filled before the run is started! ***Caution:*** Do not designate a negative ("no template") control as a known standard with "**0"** concentration. • Enter the number of samples in the **Number of Samples** field (lower left corner of window) and click **OK**.
3	Click the **Done** button to return to the **Programming Screen.**

4.1.3 Preparing PCR Samples and Starting the Program

Introduction

Before running the LightCycler, prepare the samples. Preparation involves four major stages.

Stage	Description
1	Thawing the required solutions.
2	Preparing the sample mixes.
3	Loading the capillaries and placing them into the sample carousel.
4	Placing the sample carousel into the thermal chamber and starting the program.

Thawing the Solutions

Before starting the assay, thaw all the following reagents and store them on ice:

From the...	Thaw the
LightCycler - DNA Master Hybridization Probes	• LightCycler - DNA Master Hybridization Probes (vial 1, red cap) • $MgCl_2$ stock solution (vial 2, blue cap)
LightCycler - Control Kit DNA	• β-Globin Primer Mix (vial 2, white cap) • β-Globin Hybridization Probe Mix (vial 3, yellow cap) • H_2O, sterile, PCR grade (vial 4, colorless cap)

Setting Up Capillaries

Place LightCycler capillaries into precooled centrifuge adapters. Use one capillary for every sample to be analyzed.
Note: Do not touch the surface of the capillaries with bare fingers. Always wear gloves when handling the capillaries.

Preparing a Dilution Series of the Control

- On the day of the run, prepare a dilution series of the human genomic DNA (LightCycler – Control DNA Kit, vial 1, purple cap, 15 ng/µl) in water (vial 4, colorless cap) as follows:

  1:10 = 1.5 ng/µl
  1:100 = 150 pg/µl
  1:1000 = 15 pg/µl
  1:10 000 = 1.5 pg/µl

- For PCR samples, use 2 µl of each dilution (including the undiluted DNA) as templates.

Continued on next page

4.1.3 Preparing PCR Samples and Starting the Program, Continued

Negative Control

To prepare a negative control, replace the template DNA with PCR grade water (vial 4, colorless cap) in a reaction mix containing all other reaction components.
Note: Always run a negative control with the samples!

Preparation of the Sample Mixes

Prepare PCR samples as follows:
Note: This procedure is designed for a 20 µl standard reaction.

Step	Action
1	In a 1.5 ml reaction tube on ice, prepare enough master mix for all samples to be analyzed. Add the components in the order given below. ***Note:*** The table below lists the amounts of each component needed for one reaction. To determine the total volume of each component needed, multiply the amount in the "Volume" column by (n+1), where n = the number of reactions to be cycled.
2	• Pipet 18 µl master mix into each precooled capillary. • Add 2 µl of the appropriate DNA template (undiluted or pre-diluted).

Component	Volume	Final conc.
H_2O, sterile, PCR grade (vial 4, colorless cap)	9.6 µl	
$MgCl_2$ stock solution (vial 2, blue cap)	2.4 µl	4 mM
β-Globin Primer Mix (vial 2, white cap)	2 µl	0.5 µM
β-Globin Hybridization Probe Mix (vial 3, yellow cap)	2 µl	• Probe 1: 0.2 µM • Probe 2: 0.4 µM
LightCycler - DNA Master Hybridization Probes (vial 1, red cap)	2 µl	1x
Total volume	**18 µl**	

Continued on next page

4.1.3 Preparing PCR Samples and Starting the Program, Continued

Preparation of the Sample Mixes **(continued)**

Step	Action
3	• Seal each capillary with a stopper and place the adapter, containing the capillary, into a standard benchtop microcentrifuge. • Centrifuge at 700 x g (3000 rpm in a standard benchtop microcentrifuge) for 5 s. ***Note:*** Arrange the centrifuge adapters, so the rotor is in balance, i.e., opposing samples should weigh the same.
4	Place the capillaries in the sample carousel of the LightCycler. ***Note:*** Samples can also be kept in the cooling block at 4°C until they are placed into the sample carousel.

Program Start

Start the run as follows:

Step	Action
1	Place the loaded sample carousel into the thermal chamber.
2	Click the **Run** button. ***Result:*** During the "Seek Process", the LightCycler will find the optimal capillary position for fluorescence detection of each individual sample and then start the programmed run.

End of Experimental Protocol

When the protocol is finished, the LightCycler Software will automatically exit the **Running Screen** and display the collected data on the **LightCycler Data Analysis (LCDA) Front Screen**.

4.1.4. Data Analysis with the Quantification Program

Starting Quantification Analysis

To start data evaluation with the **Quantification Program**, use this procedure:

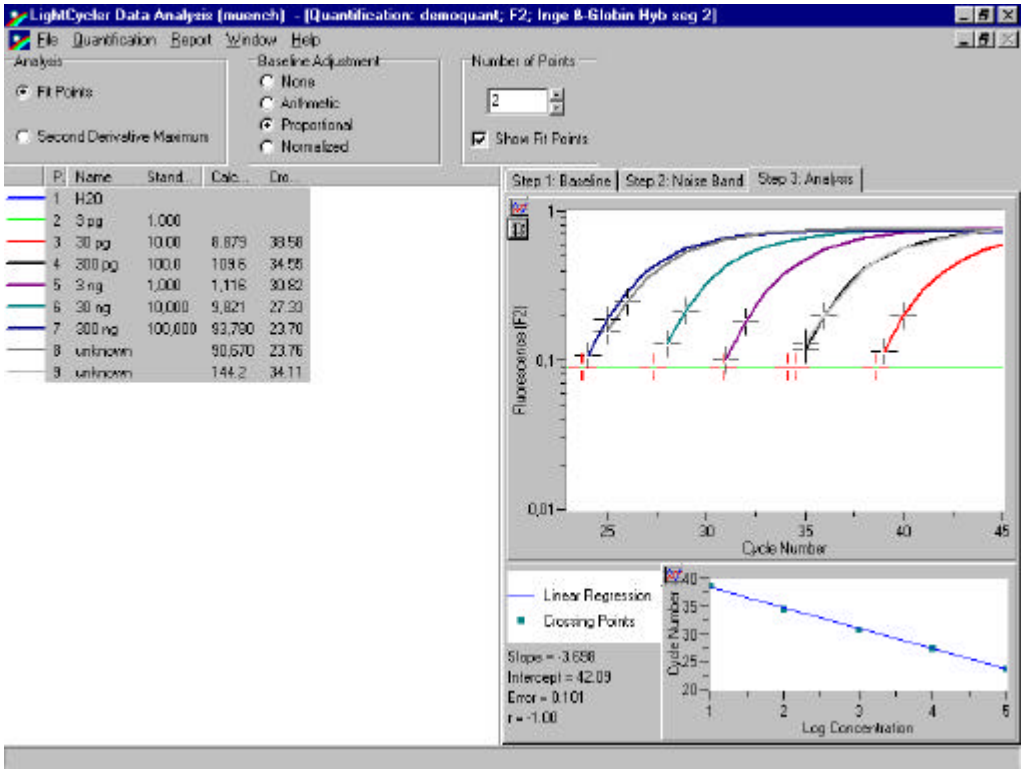
Step	Action
1	Do one of the following: • If the instrument has just finished a run and the **LCDA Front Screen** is displayed, skip Steps 2 and 3 below and go to Step 4. • If no run has been performed, (e.g. if you want to analyze data collected during a previous run), go to Step 2 below.
2	• Click on the **Data Analysis button** on the **LightCycler Front Screen**. • In the window that appears, select "Open" from the **File** menu. ***Result:*** The **Select Data File** window will open.
3	Open the appropriate run file by highlighting a file name and clicking on **Open**. ***Result:*** The **LCDA Front Screen** will appear.
4	Make sure that the amplification segment of the upper temperature chart is selected for analysis. ***Note:*** To select the amplification segment, choose the appropriate (amplification) program segment of the protocol in the **Select a Program** pull down menu.
5	Verify that the fluorescence display mode for the lower graph is **F2/F1** (in the window to the left of the graph).
6	Enter the **Quantification Screen** by clicking on the **Quantification** button (top center portion of screen).
7	Optimize the data display and analyze the data as described in Section 5 of Chapter B.

Continued on next page

4.1.4. Data Analysis with the Quantification Program, Continued

Typical Result: Fit Points

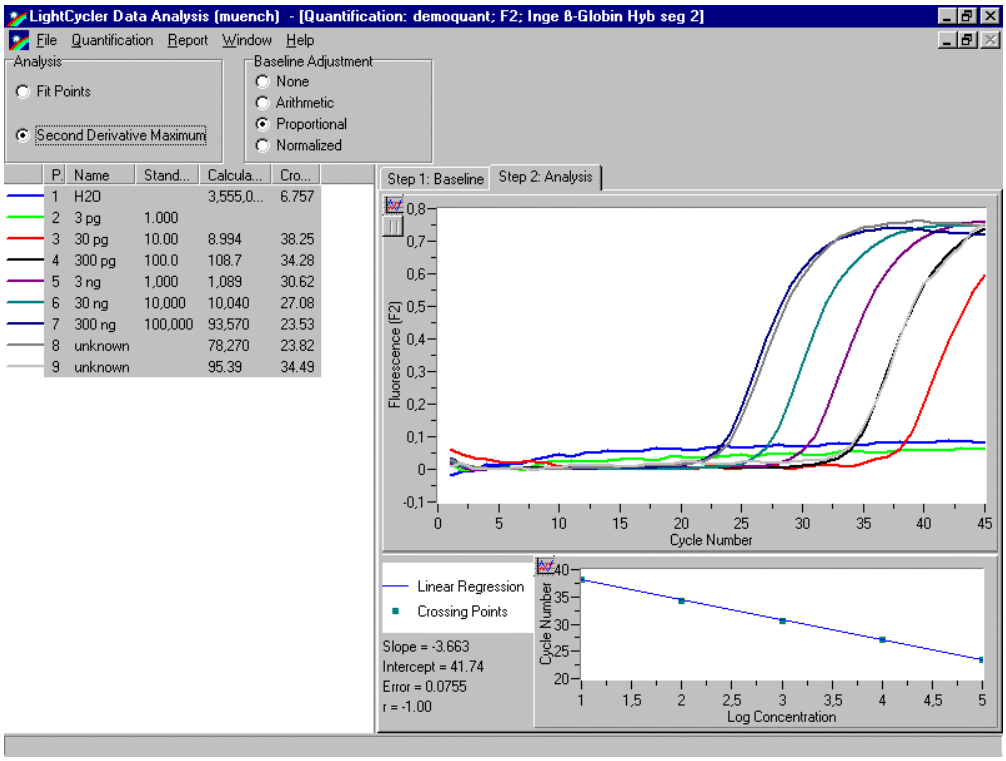
The picture below shows a quantification curve from a typical run of the procedure described in Section 4.1. The “Fit Points” method was used for analysis. The standard curve was plotted with standards containing from 10 to 1 x 10^5 gene copies.



Continued on next page

Typical Result: Second Derivative Maximum

The picture shows the same data analyzed with the Second Derivative Maximum Method.



5. Dual Color Detection with the LightCycler

5.1 Basic Information about Dual Color Detection

Introduction

The LightCycler System can simultaneously analyze two different target sequences in one sample (Dual Color Detection). Such an analysis requires two sets of hybridization probes, each specific for one of the target sequences.

In addition, the 3′-oligonucleotides (acceptor oligonucleotides) in the two sets must be labeled with two different FRET acceptor dyes.

Note: The principles of hybridization probe design and signal generation have been described elsewhere. For more information, see Hybridization Probe Design, Section 1.3.2.1 in Chapter C.

Dyes and Detection Channels

For Dual Color Detection, one of the two hybridization probe sets uses LightCycler-Red 640 (LC-Red 640, Cat. No. 2 015 161) as FRET acceptor dye; the other uses LightCycler-Red 705 (LC-Red 705, Cat. No. 2 157 594). Both acceptor dyes are excited by fluorescein. In addition, these red dyes have different emission spectra, which allows detection of a distinct signal from each set.

The dye signals generated during a run are analyzed in fluorimeter channels 2 and 3. While channel 2 (F2, 640 ± 30 nm) is measuring the fluorescence generated by LC-Red 640, channel 3 (F3, 710 ± 40 nm) is measuring LC-Red 705 signals. Fluorescent data are collected during the run from all channels and later analyzed with LightCycler Data Analysis (LCDA) software.

Applications

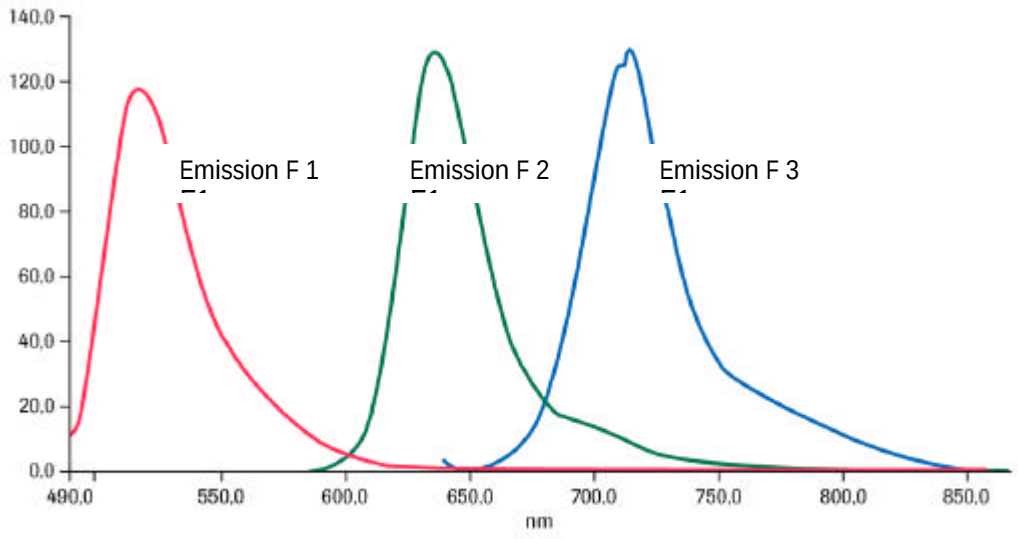
Dual Color Detection may be used for analysis of internal controls, interpretation of duplex PCR runs, or extended mutation analysis.

Continued on next page

5.1 Basic Information about Dual Color Detection, Continued

Crosstalk

Due to the spectral overlap of the emission spectra of the three dyes (as shown below), there is residual crosstalk between channels 2 and 3; some of the emitted light from LightCycler-Red 640 spills into channel 3.



Color Compensation

To correct for channel 2-channel 3 crosstalk, “color compensation” files are generated in a separate run. These files may be used in subsequent runs to correct raw (measured) fluorescence data. This color compensation feature of the LightCycler Software allows direct correlation of the fluorescent signals with the amount of hybridized probes.

Note: This chapter provides a detailed description and protocol for color compensation.

5.2 Color Calibration Test and Generation of a Color Compensation File

Background

The color compensation feature of the LightCycler Software can even out and correct for crosstalk between different channels. This crosstalk is caused by the overlap of the fluorescence emission spectra of the two red dyes used (see "Crosstalk" above).
The fluorescence emitted from LightCycler-Red 640 is measured not only in channel 2, but also partly in channel 3. Color compensation with the LightCycler Software corrects mainly for this overlap of the channel 2 signal into channel 3.
Note: Minor crosstalk also occurs from channel 3 (LightCycler-Red 705 fluorescence) into channel 2 and from channel 1 (fluorescein) into channels 2 and 3.

Factors Influencing Crosstalk

The crosstalk value is influenced by several factors:

- The emitted fluorescence intensity and spectrum of a fluorescent dye are strongly temperature dependent. The temperature chosen for data acquisition during a LightCycler PCR run influences the crosstalk significantly.
- Minor variations in the hardware component of each instrument (optical system of the fluorimeter filters, lenses and dichroic mirrors) influence the crosstalk value.

Color Compensation File

A color compensation file neutralizes the background caused by crosstalk. Instrument-specific and temperature-dependent measurements must be made every 6 months to generate the compensation file. The instrument-specific file may be generated with the help of the LightCycler Color Compensation Kit (Cat. No. 2 158 850).
After it is generated, the file may be loaded and integrated into the Data Analysis Software to correct the data for all Dual Color Detection experiments.

5.2.1 Test Overview

Overview

The procedure below describes how to perform the Color Compensation Test with the LightCycler Color Compensation Set (2 158 850).
The test simulates, in a defined protocol, all temperature conditions which may occur during a Hybridization Probe PCR protocol. All three fluorophores involved in Dual Color Detection (fluorescein, LC-Red 640, LC-Red 705) are used in the calibration test.

Test Principle

The procedure to generate a color compensation file has three stages:

Stage	Description
1	A calibration run is performed with the labeled oligonucleotides supplied in the LightCycler Color Compensation Set.
2	Instrument- and temperature-specific crosstalk data acquired in the calibration run are stored in a color compensation file.
3	The color compensation file is used for the analysis of subsequent Dual Color Detection runs.

Assay Time

The total assay time is approx. 50 minutes The assay includes the following:

Step	Time
Calibration experiment setup	5 min
LightCycler calibration run	40 min
Generation of a ccc file	5 min

Additional Material Required

To perform this assay you will need a standard benchtop microcentrifuge with a rotor for 2.0 ml tubes.
Note: Centrifuge adapters supplied with the LightCycler instrument fit into a standard microcentrifuge rotor which holds 2 ml reaction tubes.

5.2.2 Programming the Experimental Protocol

Overview

The Experimental Protocol contains four cycle programs:

- Program 1: Heating step
- Program 2: Cycling step
- Program 3: Temperature gradient step
- Program 4: Cooling step

Program 1: Heating

Set up the program for the initial heating step as follows:

Step	Action
1	From the **Front Screen,** click on the **Run** button to access the **Programming Screen**.
2	• Create a new Experimental Protocol by clicking on the **New Experiment** button in the **Experiment/RUN/Exit** window. ***Result:*** The computer will now ask for name and path of the Experimental Protocol to be programmed. • Type a file name, e.g. CC, and specify the file path. • Click **OK**
3	In the **Cycle Program** list (in the upper right part of the screen), click on the **Add** button, type the name of the program, and click **OK**.
4	In the **Cycle Program Data** and **Temperature Targets** windows, enter the following program parameters:

Parameter	Value
Cycles	1
Analysis Mode	None
Temperature Targets	***Segment 1*** ***Note:*** *Click on* ***INS*** *to insert each new segment!*
Target Temperature (°C)	95
Incubation Time (h:min:s)	30
Temperature Transition Rate (°C/s)	20.0
Secondary Target Temperature (°C)	0
Step Size (°C)	0
Step Delay (Cycle)	0
Acquisition Mode	None

Result: The program is saved automatically.

Continued on next page

5.2.2 Programming the Experimental Protocol, Continued

Program 2: Cycling

As in normal Hybridization Probe runs, the fluorescent signals are measured (acquired) at the end of the annealing phase. Set up the cycling program as follows:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>• To add a new program, click on the last program in the Cycle Program list, then click on Add.
• Type the name of the program, then click OK.</td></tr>
<tr><td>2</td><td>In the Cycle Program Data and Temperature Targets windows, enter the following program parameters:
<table>
<tr><th>Parameter</th><th colspan="3">Value</th></tr>
<tr><td>Cycles</td><td colspan="3">40</td></tr>
<tr><td>Analysis Mode</td><td colspan="3">None</td></tr>
<tr><td>Temperature Targets</td><td>Segment 1</td><td>Segment 2</td><td>Segment 3</td></tr>
<tr><td colspan="4"><u>Note:</u> Click on INS to insert each new segment!</td></tr>
<tr><td>Target Temperature (°C)</td><td>95</td><td>55</td><td>72</td></tr>
<tr><td>Incubation Time (h:min:s)</td><td>0</td><td>10</td><td>10</td></tr>
<tr><td>Temperature Transition Rate (°C/s)</td><td>20.0</td><td>20.0</td><td>20.0</td></tr>
<tr><td>Secondary Target Temperature (°C)</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Step Size (°C)</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Step Delay (cycles)</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Acquisition Mode</td><td>None</td><td>Single</td><td>None</td></tr>
</table>
<u>Result:</u> The program is saved automatically.</td></tr>
</table>

Continued on next page

5.2.2 Programming the Experimental Protocol, Continued

Program 3: Temperature Gradient

Set up the program for the temperature gradient step as follows:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>• To add a new program, click on the last program in the Cycle Program list, then click on Add.
• Type the name of the program, then click OK.</td></tr>
<tr><td>2</td><td>In the Cycle Program Data and Temperature Targets windows, enter the following program parameters:
<table>
<tr><th>Parameter</th><th colspan="3">Value</th></tr>
<tr><td>Cycles</td><td colspan="3">1</td></tr>
<tr><td>Analysis Mode</td><td colspan="3">Melting Curve</td></tr>
<tr><td>Temperature Targets</td><td>Segment 1</td><td>Segment 2</td><td>Segment 3</td></tr>
<tr><td colspan="4"><u>Note:</u> Click on INS to insert each new segment!</td></tr>
<tr><td>Target Temperature (°C)</td><td>95</td><td>55</td><td>95</td></tr>
<tr><td>Incubation Time (h:min:s)</td><td>0</td><td>30</td><td>0</td></tr>
<tr><td>Temperature Transition Rate (°C/s)</td><td>20.0</td><td>20.0</td><td>0.2</td></tr>
<tr><td>Secondary Target Temperature (°C)</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Step Size (°C)</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Step Delay (cycles)</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Acquisition Mode</td><td>None</td><td>None</td><td>Cont.</td></tr>
</table>
<u>Result:</u> The program is saved automatically.</td></tr>
</table>

Continued on next page

Program 4: Cooling Step

Before removing the rotor from the instrument, you should cool it. Set up the program for cooling the rotor and thermal chamber as follows:

Step	Action
1	• To add a new program, click on the last program in the **Cycle Program** list, then click on **Add**. • Type the name of the program, then click **OK**.
2	In the **Cycle Program Data** and **Temperature Targets** windows, enter the following program parameters: **Parameter** / **Value** Cycles: 1 Analysis Mode: None ***Temperature Targets*** / ***Segment 1*** ***Note:*** *Click on* ***INS*** *to insert each new segment!* Target Temperature (°C): 40 Incubation Time (h:min:s): 30 Temperature Transition Rate (°C/s): 20.0 Secondary Target Temperature (°C): 0 Step Size (°C): 0 Step Delay (Cycle): 0 Acquisition Mode: None ***Result:*** The program is saved automatically.
3	Save the whole experimental protocol by clicking on the **Save Experiment File** button in the **Experiment/RUN/Exit** window. If you have not already done so, enter a file name.

Setting the Fluorescence Parameters

In the **Display Mode/Fluorimeter Gains** window (upper middle part of screen), set the fluorescence parameters as follows:

- Display Mode: Choose any fluorescence channel. Data are acquired for all channels; you can switch between the channels during or after the run.
- Set the fluorescence gains as follows:

Fluorimeter Gain	Value
F1	1
F2	15
F3	30

Note: Do not select any ccc file from the "Choose ccc File" menu (upper left corner of screen)!

Continued on next page

5.2.2 Programming the Experimental Protocol, Continued

Naming of Samples

After the temperature profile is programmed, name the samples as follows:

Step	Action
1	Click on the **Edit Samples** button in the **Experiment/RUN/Exit** window. The **Loading Screen** will appear.

2	• In the **Sample Data Table** that appears, enter the following sample descriptions:
	  • Position 1: Blank
	  • Position 2: Fluorescein Calibrator
	  • Position 3: LC-Red 640 Calibrator
	  • Position 4: LC-Red 705 Calibrator.
	  Note: Do not change the order of the calibration solutions in the rotor!
	• Enter **4** in the **Number of Samples** field and click **OK**.

2	Click the **Done** button to return to the **Programming Screen**. ***Note:*** The programmed sample pattern will now be associated with this experiment and can be reused in future experiments.

5.2.3 Preparing the Calibration Samples

Overview

Before running the LightCycler, prepare the calibration reagents. Preparation involves three major stages:

Stage	Description
1	Thawing the four solutions.
2	Loading the capillaries.
3	Placing the capillaries into the sample carousel and starting the run.

Thawing

Thaw all four calibration reagents and store them on ice.

Loading the Capillaries

Proceed as follows to load the capillaries:

Step	Action
1	Place four capillaries into precooled adapters. ***Caution:*** Do not touch the surface of the capillaries with bare fingers. Always wear gloves when handling the capillaries.
2	Pipet 20 µl of each set component into a separate precooled LightCycler capillary. ***Caution:*** Do not use less than 20 µl of each sample!
3	Seal each capillary with a stopper and place the adapter, containing the capillary, into a standard benchtop microcentrifuge.
4	Centrifuge at 700 x g (3000 rpm in a standard benchtop microcentrifuge) for 5 s. ***Note:*** Arrange the centrifuge adapters so that the rotor is in balance, i.e., opposing samples should weigh the same.

Continued on next page

Starting the Run

Start the run as follows:

Step	Action
1	Place the capillaries into the rotor of the LightCycler Instrument in the following order: • Rotor position 1: Blank (vial 1) • Rotor position 2: Fluorescein Calibrator (vial 2) • Rotor position 3: LC-Red 640 Calibrator (vial 3) • Rotor position 4: LC-Red 705 Calibrator (vial 4). ***Caution:*** Do not change the order of the calibration solutions in the rotor.
2	To start the run: • Place the loaded sample carousel into the thermal chamber. • Click the **Run** button. ***Result:*** The LightCycler first seeks the optimal capillary position for fluorescence detection of each sample ("Seek Process") and then starts thermal cycling.

5.2.4 Converting the Calibration Data to a Color Compensation File

Background

After completion of the calibration run, the LightCycler software saves the generated data in a normal run file. To use these data for color compensation of experimental data, you must convert it into a **ccc** file.

Procedure

After the calibration run is complete, the **LightCycler Data Analysis (LCDA) Front Screen** appears automatically.
From the **LCDA Front Screen**, convert the color compensation data into a **ccc** file as follows:

Step	Action
1	In the **Select a Program** pull down menu, highlight the program segment that includes the melting program.
2	• From the **Color Compensation** menu, select "Calibration"**.** • Save the color compensation data by naming the calibration file. ***Note:*** The file name must end in ".ccc", e.g. data1.ccc.

5.2.5 Color Compensation of Dual Color Detection Data

Background

Dual Color Detection experiments have to be corrected for crosstalk with the color compensation files. For this purpose, the data of the most recent color compensation experiment have to be loaded into the LightCycler Data Analysis software. The software will then calculate and display the corrected data.

Note: The color compensation files can also be used to correct the fluorescence crosstalk online (see Section 5.2.6 in this chapter). Data are then displayed in the corrected mode, but stored without correction.

Procedure

To use the stored color compensation file for data compensation, proceed as follows:

Step	Action
1	• Click on the **Data Analysis** button on the **LightCycler Front Screen**. • In the window that appears, select "Open" from the **File** menu. ***Result:*** The **Select Data File** window will open. • From the **Select Data File** window, highlight and open the data file to be corrected with the LightCycler Data Analysis software. ***Result:*** The **LCDA Front Screen** will appear.
2	From the **LCDA Front Screen**, open the **Select a Program** menu and highlight the program segment to be analyzed.
3	Select "Load Calibration Data" in the **Color Compensation** menu and highlight the relevant color compensation (**ccc**) file, or
4	Click on the **Color Compensation** button (upper portion of the screen) to see the compensated data. ***Note:*** To return to the raw data, click on the **Color Compensation** button again.

5.2.6 Online Color Compensation

Procedure To view the effect of color compensation during a run, proceed as follows:

Step	Action
1	In the **Programming Screen**, open the **exp** file (i.e., filename.exp).
2	Click on **Choose CCC File** and select the appropriate color compensation file.
3	Click on **Use Color Compensation** to enable color compensation during the online display of data (on the Run Screen).

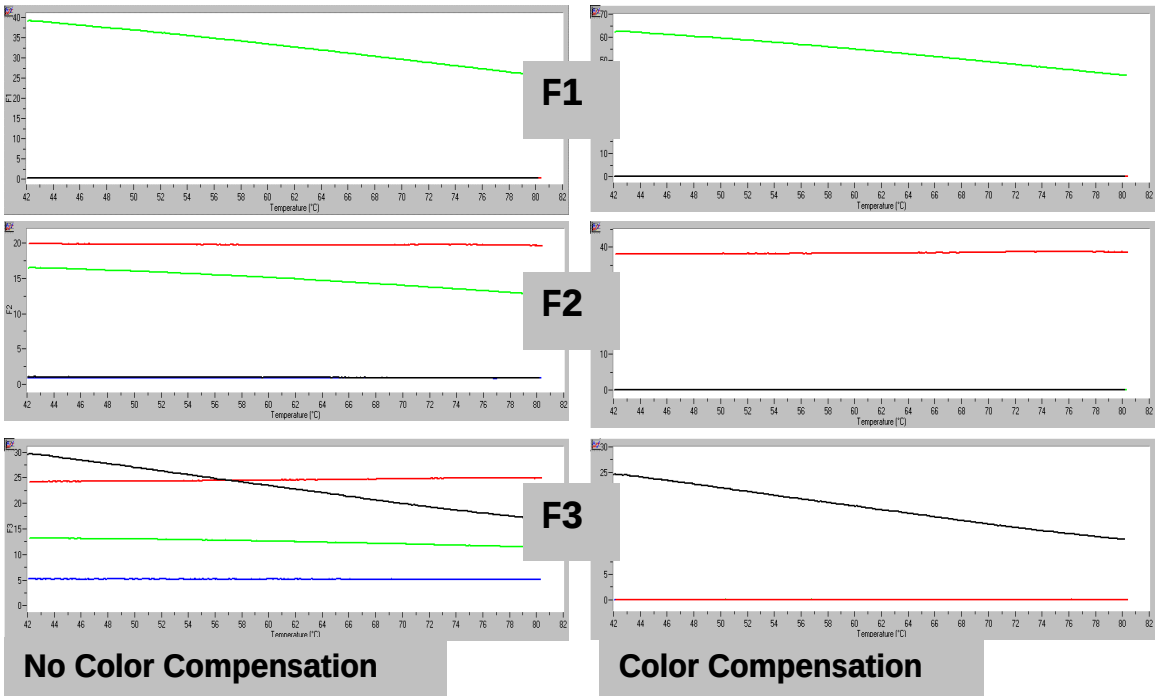
Note:

- During the run, the Display of Fluorescence History will not switch the displayed fluorescence values back to the uncorrected values if you turn color compensation off.
- Data will be stored without color compensation after the run.

5.2.7 Typical results: Calibration Run

Calibration Run Data

The figure below shows the “on screen” appearance of the fluorescence data generated during a calibration run. Fluorescence data in channels 1, 2 and 3 are displayed without compensation (left column) and with color compensation (right column).
Note: The **ccc** file used for color compensation was generated in a previous calibration run on this instrument.

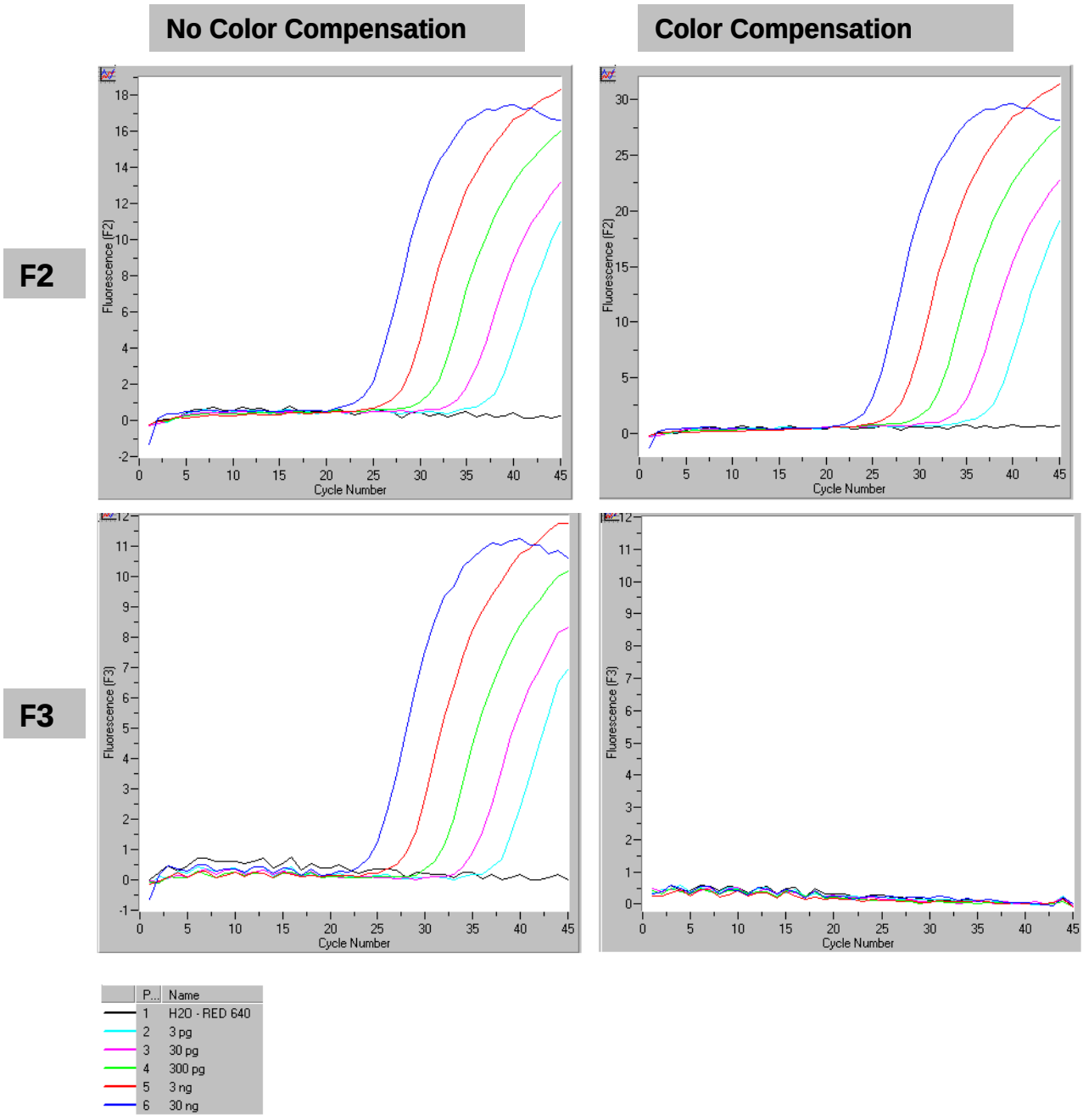


Result: All three dyes (green line: Fluorescein, red line: LightCycler Red640, black line: LightCycler Red705) are differently affected by the temperature as can be seen clearly in the left column.
While the value for the channel-specific fluorescence is enhanced after color compensation, nonspecific fluorescence is markedly diminished (right column). Note especially the reduction of fluorescence crosstalk from channel 1 and channel 2 into channel 3.

5.2.8 Typical Results: Detection of Two Target DNA Segments

Data Analysis With and Without Color Compensation

The figure below shows fluorescence data gathered during detection of different dilutions of β-Globin. The target is detected with LC-Red 640-labeled hybridization probes.



Continued on next page

5.2.8 Typical Results: Detection of Two Target DNA Segments, Continued

Data Analysis With and Without Color Compensation **(continued)**

Result: Without color compensation (left panel), fluorescence data in channel 3 show a significant increase although only LightCycler Red640 has been used as a label. After color compensation, the signal in channel 3 which was derived only from the crosstalk of the LightCycler Red640 is eliminated.

5.3 Dual Color Applications: Mutation Detection

Background

Dual Color Detection may be used to detect two different target sequences in one PCR reaction. This detection method can thus be used to analyze genes with two different mutation sites. The two sites might be detected either within two amplicons (during duplex PCR) or within one amplicon.
This chapter details the application of the Dual Color Detection method to the simultaneous detection of two point mutations within one amplicon.
Note: An example of the single color detection method, along with the design of mutation probes and anchor probes, is described in Section 6.2 of this chapter.

Genotyping

Genotyping (that is, determining the genetic makeup of an individual) may include determining the presence or absence of a mutated gene.
Several debilitating disorders are associated with single or multiple polymorphic sites. The presence of these sites indicates the individual is at risk to develop the disorder. In the model system described below, the mutation sites may be found within a single exon and therefore may be detected within one amplicon.

Detection Format

This experiment required the design of two sets of hybridization probes, which span the mutated region. Probe sequences matched the wild type sequence.
The two "mutation" probes (i.e., the hybridization probes covering the actual mutation sites) were labeled with LightCycler-Red 640 (LC-Red 640) and LC-Red 705, respectively. The two fluorescent signals generated in the melting curve analysis thus provide information about the presence or absence of the two mutant sequences.

Samples

Detection of two different mutation sites was performed for two samples:

- Human genomic DNA with a known genotype, and
- Plasmid preparation containing the following sequence mixtures:

Mutation Position 1 (LC-Red 640)			**Mutation Position 2 (LC-Red 705)**		
No	Type	Sequence Allele 1/Allele 2	No	Type	Sequence Allele 1/Allele 2
1	Homozygous Wild Type	CGC/CGC	4	Homozygous Wild Type	CGC/CGC
2	Heterozygous	CGC/TGC	5	Heterozygous	CGC/TGC
3	Homozygous Mutant	TGC/TGC	6	Homozygous Mutant	TGC/TGC
			7	No template control	(NTC)

Continued on next page

5.3 Dual Color Applications: Mutation Detection, Continued

Hybridization Probes Design

The hybridization probes have the following features:

	Anchor Probe	Mutation Probe
Position 1	• Length: 25 bases • Labeled with fluorescein	• Length: 19 bases • Labeled with LC-Red 640
Position 2	• Length: 29 bases • Labeled with fluorescein	• Length: 20 bases • Labeled with LC-Red 705

Test principle

The procedure for Dual Color Detection of mutations has two main stages:

Step	Action
1	LightCycler PCR with subsequent melting curve analysis.
2	Analysis of data (color compensation and melting curve analysis).

5.3.1 Programming the Experimental Protocol

Test Overview

The Experimental Protocol for the Tests contains four cycle programs:
Program 1: Denaturation of the template DNA
Program 2: Amplification of the target DNAs
Program 3: Melting Curve analysis (for mutation detection)
Program 4: Cooling of the instrument.

Program 1: Denaturation

Set up the program for the denaturation step as follows:

Step	Action
1	From the **Front Screen,** click on the **Run** button to access the **Programming Screen**.
2	• Create a new Experimental Protocol by clicking on the **New Experiment** button in the **Experiment/RUN/Exit** window. ***Result:*** The computer will now ask for name and path of the Experimental Protocol to be programmed. • Type a file name and specify the file path. • Click **OK**
3	In the **Cycle Program** list (in the upper right part of the screen), click on the **Add** button, type the name of the program, and click **OK**.
4	In the **Cycle Program Data** and **Temperature Targets** windows, enter the following program parameters: **Parameter** / **Value** Cycles: 1 Analysis Mode: None ***Temperature Targets*** / ***Segment 1*** ***Note:*** *Click on* ***INS*** *to insert each new segment* Target Temperature (°C): 95 Incubation Time (s): 30 Temperature Transition Rate (°C/s): 20.0 Secondary Target Temperature (°C): 0 Step Delay (Cycles): 0 Acquisition Mode: None ***Result:*** The program is saved automatically.

Continued on next page

Program 2: Amplification

During amplification, the fluorescent signal is measured (acquired) at the end of the elongation phase. Set up the amplification program as follows:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>• To add a new program, click on the last program in the Cycle Program list, then click on Add.
• Type the name of the program, then click OK.</td></tr>
<tr><td>2</td><td>In the Cycle Program Data and Temperature Targets windows, enter the following program parameters:
<table>
<tr><th>Parameter</th><th colspan="3">Value</th></tr>
<tr><td>Cycles</td><td colspan="3">45</td></tr>
<tr><td>Analysis Mode</td><td colspan="3">Quantification</td></tr>
<tr><td>Temperature Targets</td><td>Segment 1</td><td>Segment 2</td><td>Segment 3</td></tr>
<tr><td colspan="4"><u>Note:</u> Click on INS to insert each new segment!</td></tr>
<tr><td>Target Temperature (°C)</td><td>95</td><td>60</td><td>72</td></tr>
<tr><td>Incubation Time (h:min:s)</td><td>0</td><td>10</td><td>15</td></tr>
<tr><td>Temperature Transition Rate (°C/s)</td><td>20.0</td><td>20.0</td><td>20.0</td></tr>
<tr><td>Secondary Target Temperature (°C)</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Step Size (°C)</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Step Delay (cycles)</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Acquisition Mode</td><td>None</td><td>Single</td><td>None</td></tr>
</table>
<u>Result:</u> The program is saved automatically.</td></tr>
</table>

Continued on next page

Program 3: Melting Curve Analysis

Set up the program for the melting curve analysis as follows:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>• To add a new program, click on the last program in the Cycle Program list, then click on Add.
• Type the name of the program, then click OK.</td></tr>
<tr><td>2</td><td>In the Cycle Program Data and Temperature Targets windows, enter the following program parameters:
<table>
<tr><th>Parameter</th><th colspan="3">Value</th></tr>
<tr><td>Cycles</td><td colspan="3">45</td></tr>
<tr><td>Analysis Mode</td><td colspan="3">Melting Curves</td></tr>
<tr><td>Temperature Targets</td><td>Segment 1</td><td>Segment 2</td><td>Segment 3</td></tr>
<tr><td></td><td colspan="3"><u>Note:</u> Click on INS to insert each new segment!</td></tr>
<tr><td>Target Temperature (°C)</td><td>95</td><td>42</td><td>80</td></tr>
<tr><td>Incubation Time (h:min:s)</td><td>30</td><td>2:00</td><td>0</td></tr>
<tr><td>Temperature Transition Rate (°C/s)</td><td>20.0</td><td>20.0</td><td>0.1</td></tr>
<tr><td>Secondary Target Temperature (°C)</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Step Size (°C)</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Step Delay (cycles)</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Acquisition Mode</td><td>None</td><td>None</td><td>Cont.</td></tr>
</table>
<u>Result:</u> The program is saved automatically.</td></tr>
</table>

Continued on next page

Program 4: Cooling

Before removing the rotor from the instrument, you should cool it. Set up the program for cooling the rotor and thermal chamber as follows:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>• To add a new program, click on the last program in the Cycle Program list, then click on Add.
• Type the name of the program, then click OK.</td></tr>
<tr><td>2</td><td>In the Cycle Program Data and Temperature Targets windows, enter the following program parameters:

<table>
<tr><th>Parameter</th><th>Value</th></tr>
<tr><td>Cycles</td><td>1</td></tr>
<tr><td>Analysis Mode</td><td>None</td></tr>
<tr><td>Temperature Targets</td><td>Segment 1
<u>Note:</u> Click on INS to insert each new segment!</td></tr>
<tr><td>Target Temperature (°C)</td><td>40</td></tr>
<tr><td>Incubation Time (h:min:s)</td><td>30</td></tr>
<tr><td>Temperature Transition Rate (°C/s)</td><td>20.0</td></tr>
<tr><td>Secondary Target Temperature (°C)</td><td>0</td></tr>
<tr><td>Step Size (°C)</td><td>0</td></tr>
<tr><td>Step Delay (Cycle)</td><td>0</td></tr>
<tr><td>Acquisition Mode</td><td>None</td></tr>
</table>

<u>Result:</u> The program is saved automatically.</td></tr>
<tr><td>3</td><td>Save the whole experimental protocol by clicking on the Save Experiment File button in the Experiment/RUN/Exit window. If you have not already done so, enter a file name.</td></tr>
</table>

Naming of Samples

Click on the **Edit Samples** button and enter the appropriate names and concentrations for each sample, along with the number of samples in the **Loading Screen** (as described in Section 2.2.2 of this chapter).

5.3.2 Preparing PCR Samples and Starting the Run

Preparation of Samples

Prepare the Dual Color Detection Samples as follows:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>In a 1.5 ml reaction tube on ice, prepare enough master mix for all samples to be analyzed. Add the components in the order given below.
<u>Note:</u> The table below lists the amounts of each component needed for one reaction. To determine the total volume of each component needed, multiply the amount in the “Volume” column by (n+1), where n = the number of reactions to be cycled.
<table>
<tr><th>Component</th><th>Volume</th><th>Final conc.</th></tr>
<tr><td>H_2O, sterile, PCR grade (vial 4, colorless cap</td><td>To 18 µl (final volume)</td><td></td></tr>
<tr><td>$MgCl_2$ stock solution (vial 2, blue cap)</td><td>w µl</td><td>1–5 mM *</td></tr>
<tr><td>Primers</td><td>x µl **</td><td>0.3–1 µM each *</td></tr>
<tr><td>Hybridization Probes Set Position 1</td><td>y µl **</td><td>0.2 µM each</td></tr>
<tr><td>Hybridization Probes Set Position 2</td><td>z µl **</td><td>0.2 µM each</td></tr>
<tr><td>LightCycler-DNA Master Hybridization Probes (vial 1, red cap)</td><td>2 µl</td><td>1x</td></tr>
<tr><td>Internal Control</td><td>1 µl</td><td></td></tr>
<tr><td>Total volume</td><td>18 µl</td><td></td></tr>
</table>
* Optimum concentration determined experimentally.
** Exact volume depends on concentration of stock solutions.</td></tr>
<tr><td>2</td><td>• Pipet 18 µl master mix into each precooled capillary.
• Add 2 µl of the appropriate pre-diluted cDNA.</td></tr>
<tr><td>3</td><td>• Seal each capillary with a stopper and place the adapter, containing the capillary, into a standard benchtop centrifuge.
• Centrifuge at 700 x g (3000 rpm in a standard benchtop centrifuge) for 5 s.</td></tr>
<tr><td>4</td><td>Place the capillaries into the rotor of the instrument.
<u>Note:</u> Samples can also be kept in the cooling block at 4°C until they are placed into the sample carousel.</td></tr>
</table>

Continued on next page

5.3.2 Preparing PCR Samples and Starting the Run, Continued

Program Start

Start the run as follows:

Step	Action
1	Place the loaded sample carousel into the thermal chamber.
2	Click the **Run** button. ***Result:*** During the "Seek Process", the LightCycler Instrument will find the optimal capillary position for fluorescence detection of each individual sample and then start the programmed run.

End of Experimental Protocol

When the protocol has finished, the LightCycler Software will automatically exit the **Running Screen** and display the collected data in the **LightCycler Data Analysis (LCDA) Front Screen**.

5.3.3 Starting Data Analysis

Starting Data Analysis

To analyze the acquired data, do the following:

Step	Action
1	Do one of the following: • If the instrument has just finished a run and the **LCDA Front Screen** is displayed, skip Steps 2 and 3 below and go to Step 4. • If no run has been performed, (e.g. if you want to analyze data collected during a previous run), go to Step 2 below.
2	• Click on the **Data Analysis** button on the **LightCycler Front Screen**. • In the window that appears, select "Open" from the **File** menu. ***Result:*** The **Select Data File** window will open.
3	Open the file you want to analyze by highlighting the name of the file and clicking on **Open**. ***Result:*** The **LCDA Front Screen** will appear.
4	To import the appropriate color compensation file: • Click on the **Select CC Data** button. • Select the most recent color compensation file.
5	Click on the **Color Compensation** button to view the compensated data

5.3.4 Typical Results: Mutation Detection

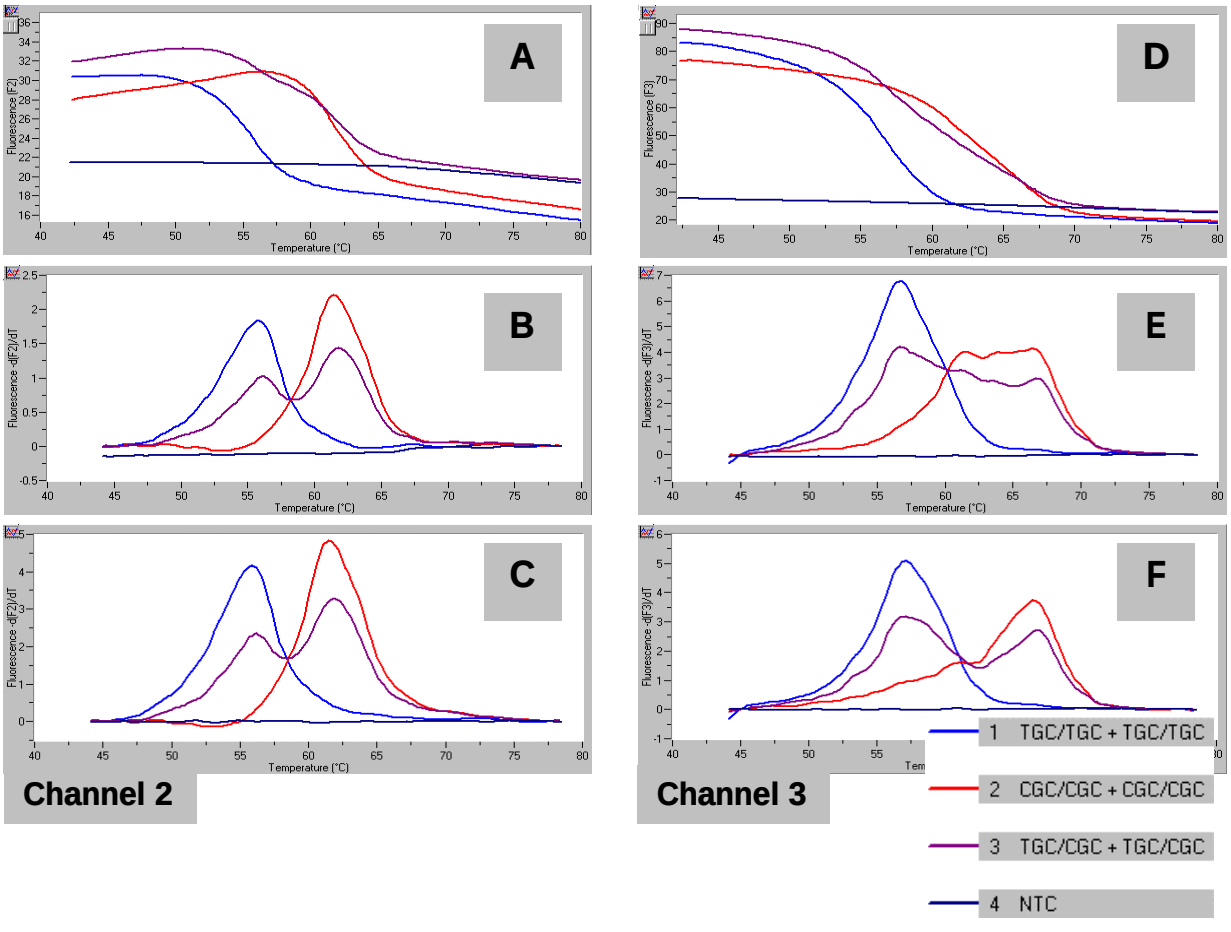
Genotyping

The Dual Color Detection protocol described in Section 5.3 was used to analyze two samples: (1) a plasmid mixture containing all possible mutation combinations and (2) a 100 ng human DNA sample of known genotype.

Typical Results with Plasmid Mixture

Analysis of the plasmid mixture yielded the results shown below. While A, B, and C (generated with LC-Red 640-labeled probes) indicate the presence of the position 1 mutation, D, E, and F (generated with LC-Red 705-labeled probes) indicate the presence of the position 2 mutation.

A	**Raw data** melting curve fluorescence detected in **channel 2** (F2)
B	**Melting peaks of channel 2** (F2) **without color compensation**.
C	Same data as displayed in B, **but with color compensation**.
D	Raw data melting curve fluorescence as detected in **channel 3** (F3).
E	**Melting peaks of channel 3** (F3).
F	Same data as displayed in E**, but with color compensation.**



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5.3.4 Typical Results: Mutation Detection, Continued

Typical Results with Plasmid Mixture **(continued)**

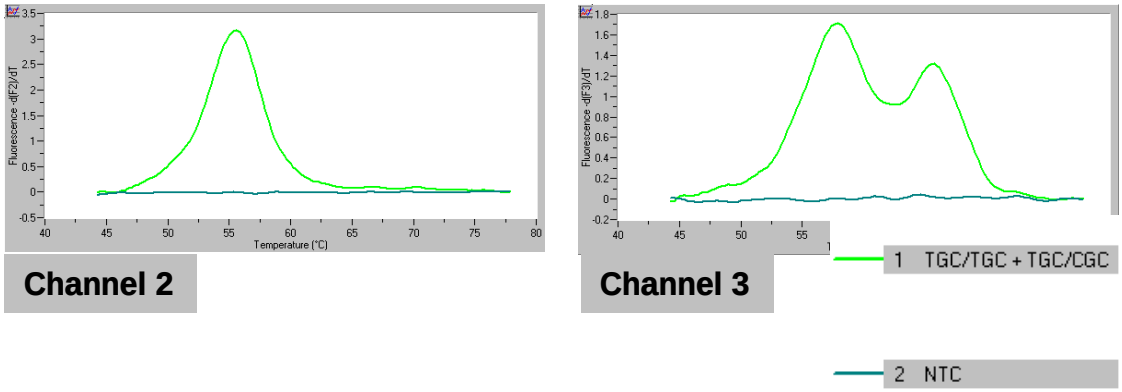
Result: For both mutation sites, homozygous wild type and mutant show one melting peak, while the heterozygous mutant shows two peaks. A comparison of channel 2 and channel 3 defines all possible combinations of the mutations.

Continued on next page

5.3.4 Typical Results: Mutation Detection, Continued

Typical Results with Human DNA Sample

The figure below shows the Dual Color Detection analysis performed on 1 ng total human DNA.



Result: The analysis reveals that the individual from whom the DNA was prepared is homozygous for the mutation in position 1 but heterozygous for the mutation in position 2.

5.4 Dual Color Applications: Simultaneous Detection of Target DNA and Internal Positive Control in one Capillary

Background

The LightCycler System, when used in Hybridization Probes format, can detect two different target DNA sequences with two sets of hybridization probes. The two sets of hybridization probes differ in the label of the 3′ probe as described above (see Section 5.1 in this chapter). The signals from these probes are detected in fluorimeter channel 2 and channel 3. This setup allows detection of specific signals for each of the target sequences; these signals can be analyzed in the Quantification and Melting Curve Analysis module.
While Section 5.3 in this chapter describes the detection of two different target sequences within one amplicon, the current section describes the amplification and detection of two related DNA sequences.

Internal Control Experiments

An internal control fragment is co-amplified with the target DNA sequence. The control shares primer binding sites with the target, but contains internal sequences that differ from the target sequence. Thus, only one pair of primers is required to amplify the target and the internal control, but two sets of hybridization probes are necessary to recognize the differences between the two DNAs.
The internal control serves as a performance indicator for the PCR. Factors, e.g. sample characteristics, that inhibit the PCR will affect not only the target sequence—where it might cause false negatives—but also the internal control fragment.

5.4.1 Test Overview

Test Principle

Equal amounts of an internal control are added to samples and standards. The procedure has three main stages:

Stage	Description
1	RT-PCR.
2	LightCycler PCR.
3	Analysis of data with the **Color Compensation** file and the **Quantification** program.

Samples

Messenger RNA from W1-38 cells was reverse transcribed with the First Strand cDNA Synthesis Kit for RT-PCR (AMV), Cat. No. 1 483 188. The resulting cDNA was amplified with tPA (tissue plasminogen activator) - specific primers. The internal control fragment was a plasmid-derived DNA sequence with the same primer binding sites as the target tPA sequence.

Hybridization Probes Design

The hybridization probes have the following features:

	5′ hybridization probe	3′ hybridization probe
Target DNA (tPA)	• Length: 22 bases • Labeled with fluorescein	• Length: 19 bases • Labeled with LC-Red 640
Internal Control	• Length: 22 bases • Labeled with fluorescein	• Length: 20 bases • Labeled with LC-Red 705

Assay time

The total assay time is approx. 135 minutes. The assay includes the following:

Step	Time
Set up and perform RT reaction	75 min
Prepare dilution series and set up samples	30 min
Amplify and analyze melting curve	30 min

Additional Material Required

- For the RT reaction: First Strand cDNA Synthesis Kit for RT-PCR (AMV), Cat. No. 1 483 188.
- Standard benchtop microcentrifuge with a rotor for 2.0 ml tubes.
  Note: Centrifuge adapters supplied with the LightCycler instrument fit into a standard microcentrifuge rotor that holds 2 ml tubes.

5.4.2. Programming the Experimental Protocol

Test Overview

The Experimental Protocol contains four cycle programs:
Program 1: Denaturation of the template DNA
Program 2: Amplification of target DNAs
Program 3: Melting Curve analysis (for detection of target and control)
Program 4: Cooling of the instrument.

Program 1: Denaturation

Set up the program for the denaturation step as follows:

Step	Action
1	From the **Front Screen,** click on the **Run** button to access the **Programming Screen**.
2	• Create a new Experimental Protocol by clicking on the **New Experiment** button in the **Experiment/RUN/Exit** window. ***Result:*** The computer will now ask for name and path of the Experimental Protocol to be programmed. • Type a file name and specify the file path. • Click **OK**
3	In the **Cycle Program** list (in the upper right part of the screen), click on the **Add** button, type the name of the program, and click **OK**.
4	In the **Cycle Program Data** and **Temperature Targets** windows, enter the following program parameters:

Parameter	Value
Cycles	1
Analysis Mode	None
Temperature Targets	***Segment 1*** ***Note:*** *Click on* ***INS*** *to insert each new segment*
Target Temperature (°C)	95
Incubation Time (h:min:s)	30
Temperature Transition Rate (°C/s)	20.0
Secondary Target Temperature (°C)	0
Step Delay (Cycles)	0
Acquisition Mode	none

Result: The program is saved automatically.

Continued on next page

5.4.2. Programming the Experimental Protocol, Continued

Program 2: Amplification

During amplification, the fluorescent signal is measured (acquired) at the end of the elongation phase. Set up the amplification program as follows:

Step	Action
1	Click **Add** to add a new cycle program.
2	Enter the following program parameters:

Parameter	**Value**		
Cycles	45		
Analysis Mode	Quantification		
Temperature Targets	**Segment 1**	**Segment 2**	**Segment 3**
	Note: *Click on* ***INS*** *to insert each new segment!*		
Target Temperature (°C)	95	58	72
Incubation Time (h:min:s)	2	10	15
Temperature Transition Rate (°C/s)	20.0	20.0	3.0
Secondary Target Temperature (°C)	0	0	0
Step Size (°C)	0	0	0
Step Delay (cycles)	0	0	0
Acquisition Mode	None	Single	None

Result: The program is saved automatically.

Continued on next page

5.4.2. Programming the Experimental Protocol, Continued

Program 3: Melting Curve Analysis

Set the program for the melting curve analysis as follows:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>• To add a new program, click on the last program in the Cycle Program list, then click on Add.
• Type the name of the program, then click OK.</td></tr>
<tr><td>2</td><td>In the Cycle Program Data and Temperature Targets windows, enter the following program parameters:
<table>
<tr><th>Parameter</th><th colspan="3">Value</th></tr>
<tr><td>Cycles</td><td colspan="3">45</td></tr>
<tr><td>Analysis Mode</td><td colspan="3">Melting Curves</td></tr>
<tr><td>Temperature Targets</td><td>Segment 1</td><td>Segment 2</td><td>Segment 3</td></tr>
<tr><td></td><td colspan="3"><u>Note:</u> Click on INS to insert each new segment!</td></tr>
<tr><td>Target Temperature (°C)</td><td>95</td><td>45</td><td>95</td></tr>
<tr><td>Incubation Time (h:min:s)</td><td>0</td><td>2:00</td><td>0</td></tr>
<tr><td>Temperature Transition Rate (°C/s)</td><td>20.0</td><td>20.0</td><td>0.1</td></tr>
<tr><td>Secondary Target Temperature (°C)</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Step Size (°C)</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Step Delay (cycles)</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Acquisition Mode</td><td>None</td><td>None</td><td>Cont.</td></tr>
</table>
<u>Result:</u> The program is saved automatically.</td></tr>
</table>

Continued on next page

Program 4: Cooling

Before removing the rotor from the instrument, you should cool it. Set up the program for cooling the rotor and thermal chamber as follows:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>• To add a new program, click on the last program in the Cycle Program list, then click on Add.
• Type the name of the program, then click OK.</td></tr>
<tr><td>2</td><td>In the Cycle Program Data and Temperature Targets windows, enter the following program parameters:

<table>
<tr><th>Parameter</th><th>Value</th></tr>
<tr><td>Cycles</td><td>1</td></tr>
<tr><td>Analysis Mode</td><td>None</td></tr>
<tr><td>Temperature Targets</td><td>Segment 1
<u>Note:</u> Click on INS to insert each new segment!</td></tr>
<tr><td>Target Temperature (°C)</td><td>40</td></tr>
<tr><td>Incubation Time (s)</td><td>30</td></tr>
<tr><td>Temperature Transition Rate (°C/s)</td><td>20.0</td></tr>
<tr><td>Secondary Target Temperature (°C)</td><td>0</td></tr>
<tr><td>Step Size (°C)</td><td>0</td></tr>
<tr><td>Step Delay (Cycle)</td><td>0</td></tr>
<tr><td>Acquisition Mode</td><td>None</td></tr>
</table>

<u>Result:</u> The program is saved automatically.</td></tr>
<tr><td>3</td><td>Save the whole experimental protocol by clicking on the Save Experiment File button in the Experiment/RUN/Exit window. If you have not already done so, enter a file name.</td></tr>
</table>

Naming of Samples

Click on the **Edit Samples** button and enter the appropriate names and concentrations for each sample and the sample number in the **Loading Screen** as described in Section 2.2.2 of this chapter.

5.4.3 Preparing PCR Samples and Starting the Program

Introduction

Before running the LightCycler Instrument, prepare the samples. Sample preparation involves three major stages:

Stage	Description
1	Perform an RT reaction to generate cDNA for the standard, the internal control, and the sample mRNA.
2	Set up the dilution series for the standards and add internal control to all samples.
3	Prepare the sample mixes

RT Reaction

Use either the First Strand cDNA Synthesis Kit for RT-PCR (Cat. No. 1 483 188) or Expand Reverse Transcriptase (Cat. No. 1 785 826)* to perform cDNA synthesis.

Note: Follow instructions given in the pack insert for the product.

A standard RT reaction involves the reverse transcription of 1 µg total RNA in a final volume of 20 µl.

Dilution Series and Internal Control

Prepare a dilution series of the target cDNA and add the appropriate internal control DNA as follows:

Step	Action
1	Prepare a fresh series of dilutions with the standard cDNA.
2	Set up a PCR reaction with 2 µl of each standard cDNA dilution.
3	Add an appropriate amount of internal control cDNA to each reaction: • Dilute the internal control cDNA so 1 µl of the control solution contains as much DNA as 2 µl of a dilution prepared in Step 1. A midrange concentration works best. (For example, if you prepared a logarithmic dilution series in which 2 µl of a dilution contained 10^2 to 10^7 copies of standard control DNA, prepare a control solution in which 1 µl contains 10^4 copies of the internal control DNA. • Add 1 µl of the diluted internal control cDNA to each PCR reaction tube.

Controls

Prepare a negative control sample containing all reaction components except the template cDNA and internal control cDNA. Replace these cDNAs with an equal volume of PCR grade water (vial 4) from the LightCycler - DNA Master Hybridization Probes (Cat. No. 2 015 102).

Note: Always include a negative control to check for the amplification of nonspecific products.

Continued on next page

* Not available in the U.S.

5.4.3 Preparing PCR Samples and Starting the Program, Continued

Preparation of Sample Mixes

Prepare the Dual Color Detection samples as follows:

Step	Action
1	In a 1.5 ml reaction tube on ice, prepare enough master mix for all samples to be analyzed. Add the components in the order given below. ***Note:*** The table below lists the amounts of each component needed for one reaction. To determine the total volume of each component needed, multiply the amount in the "Volume" column by (n+1), where n = the number of reactions to be cycled.

Component	Volume	Final conc.
H_2O, sterile, PCR grade (vial 4, colorless cap)	To 18 µl (final volume)	
$MgCl_2$ stock solution (vial 2, blue cap)	2 µl	2.5 mM
Primers	x µl *	0.5 µM each
Hybridization Probes, tPA	y µl *	0.2 µM each
Hybridization Probes, internal control	z µl *	0.2 µM each
LightCycler-DNA Master Hybridization Probes (vial 1, red cap)	2 µl	1x
Internal Control	1 µl	
Total volume	**18 µl**	

* Exact volume depends on concentration of stock solutions.

2	• Pipet 18 µl master mix into each precooled capillary. • Add 2 µl of the appropriate pre-diluted cDNA to each capillary.
3	• Seal each capillary with a stopper and place the adapter, containing the capillary, into a standard benchtop centrifuge. • Centrifuge at 700 x g (3000 rpm in a standard benchtop centrifuge) for 5 s.
4	Place the capillaries into the rotor of the instrument. ***Note:*** Samples can also be kept in the cooling block at 4°C until they are placed into the sample carousel.

Continued on next page

Program Start

Start the run as follows:

Step	Action
1	Place the loaded sample carousel into the thermal chamber.
2	Click the **Run** button. ***Result:*** During the "Seek Process", the LightCycler Instrument will find the optimal capillary position for fluorescence detection of each individual sample and then start the programmed run.

End of Experimental Protocol

When the protocol has finished, the LightCycler software will automatically exit the **Running Screen** and display the collected data in the **LightCycler Data Analysis (LCDA) Front Screen**.

Continued on next page

5.4.4 Starting Data Analysis

Starting Data Analysis

To analyze the acquired data, do the following:

Step	Action
1	Do one of the following: • If the instrument has just finished a run and the **LCDA Front Screen** is displayed, skip Steps 2 and 3 below and go to Step 4. • If no run has been performed, (e.g. if you want to analyze data collected during a previous run), go to Step 2 below.
2	• Click on the **Data Analysis** button on the **LightCycler Front Screen**. • In the window that appears, select "Open" from the **File** menu. ***Result:*** The **Select Data File** window will open.
3	Open the file you want to analyze by highlighting the name of the file and clicking on **Open**. ***Result:*** The **LCDA Front Screen** will appear.
4	To import the appropriate color compensation file: • Click on the **Select CC Data** button. • Select the most recent color compensation file.
5	Click on the **Color Compensation** button to view the compensated data.

5.4.5 Typical Results: Detection of Target and Internal Control

Detection of tPA DNA with Internal Control

Hybridization probes labeled with LightCycler-Red 640 were used to detect tPA (tissue plasminogen activator) mRNA in W1-38 cells.
A standard curve was generated from plasmid-derived tPA cDNA. The internal control was a mutated tPA fragment containing a 15-base insertion. A set of hybridization probes labeled with LightCycler-Red 705 was used to detect the internal control. In addition, the tPA-mRNA content of W1-38 cells was analyzed.

Sample Names

Samples displayed on the analysis screens were named as follows:

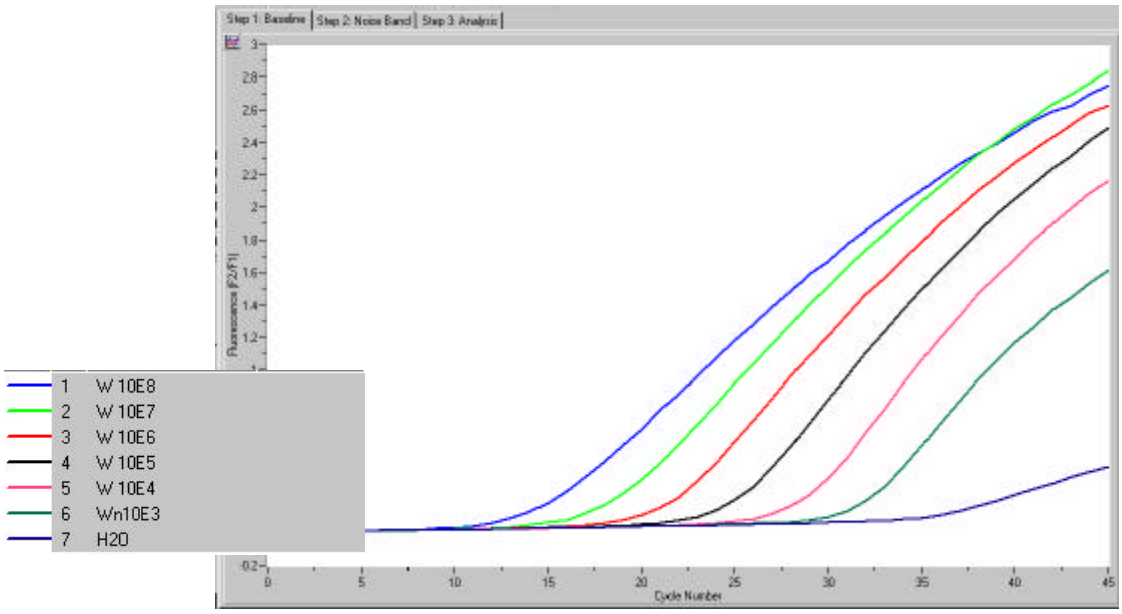
Sample Name	Content
W 10E8	Dilution series standard cDNA (10^8 copies to 10^3 copies) without internal control. Signal in channel 2.
W 10E7	
W 10E6	
W 10E5	
W 10E4	
W 10E3	
H2O	
W 10E8 +C 10E4	Dilution series standard cDNA (10^8 copies to 10^3 copies) with internal control (10^4 copies). Signal in channel 2 and channel 3.
W 10E7 +C 10E4	
W 10E6 +C 10E4	
W 10E5 +C 10E4	
W 10E4 +C 10E4	
W 10E3 +C 10E4	
H2O +C 10E4	
2.2 x 10E4 cells + C	Cellular mRNA from different numbers of cells (22000, 2200, 220 and 22 cells) with internal control.
2.2 x 10E3 cells + C	
2.2 x 10E2 cells + C	
2.2 x 10E cells + C	

Continued on next page

5.4.5 Typical Results: Detection of Target and Internal Control, Continued

Typical Results: Control Reaction

The picture below shows data from the control reaction (dilution series of standard cDNA without internal control) gathered in channel 2.



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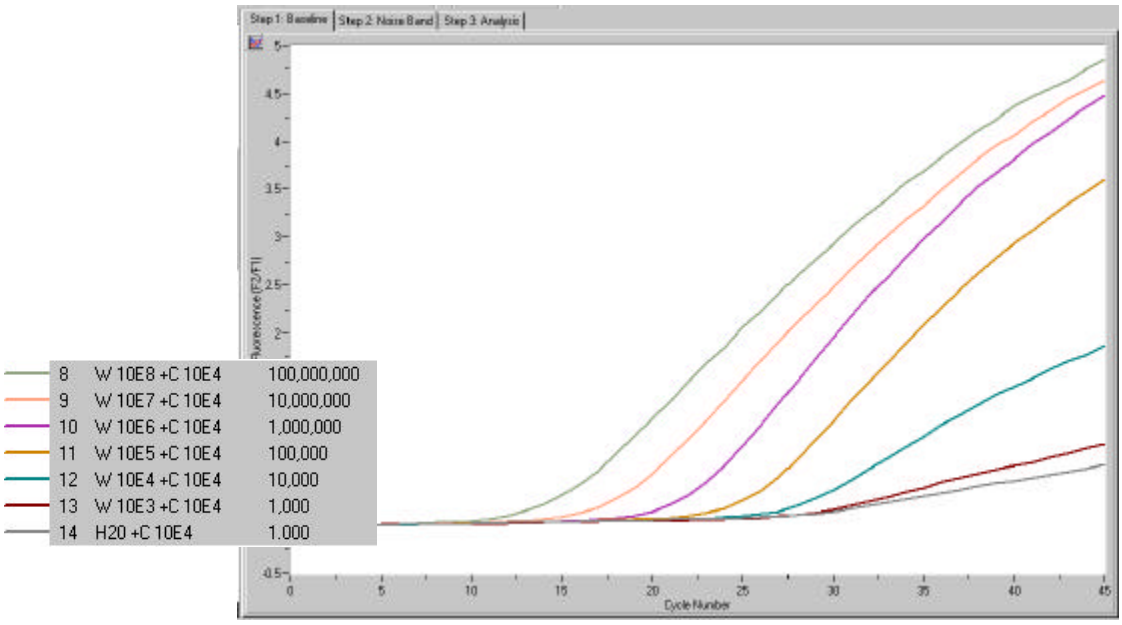
5.4.5 Typical Results: Detection of Target and Internal Control, Continued

Typical Results: Standards–Target DNA

The picture below shows the channel 2 (F2) data for the standard curve obtained in the presence of the internal control (competitor cDNA). These data represent amplification of the target DNA.

Note: Since both fragments compete for one primer pair, the concentration values at the low end of the curve are slightly lower than the corresponding values on the control standard curve (obtained without competitor. However, quantification is possible with this corrected data.



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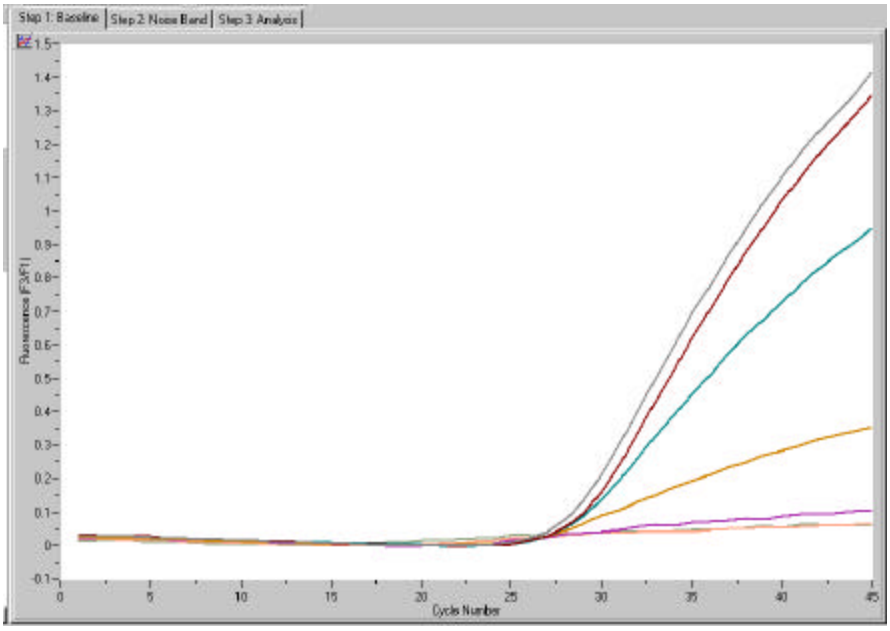
5.4.5 Typical Results: Detection of Target and Internal Control, Continued

Typical Results: Standards–Internal Control

The picture below shows the channel 3 data for the same samples as in the "Standards–Target DNA" picture on the previous page. These data represent signals from the internal competitor. Note that the threshold cycle for emerging fluorescence in channel 3 is the same for all samples, indicating that all samples from the standard curve contain the same initial amount of the internal control.

Since an increase in the amount of target DNA causes a decrease in the amplification efficiency of the internal control, the slope of the amplification curve indicates the amplification efficiency of the reaction. Thus, the lower the starting copy number for the target DNA, the higher the signal for the internal control.



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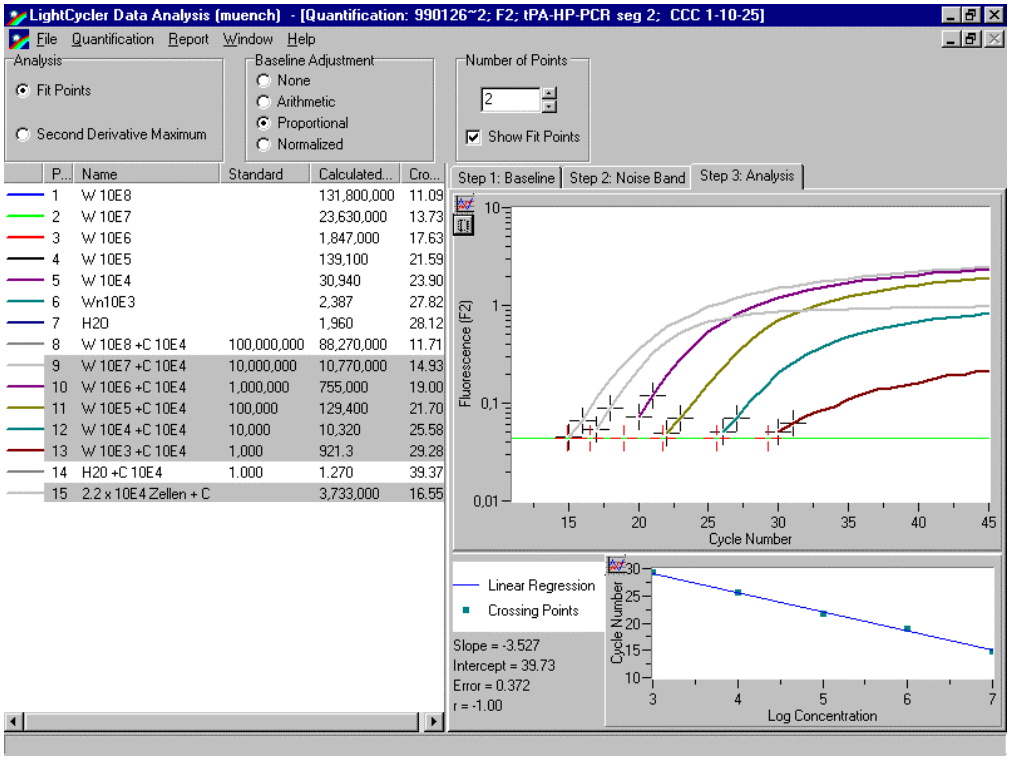
5.4.5 Typical Results: Detection of Target and Internal Control,
Continued

Typical Results: Cellular mRNA

A “Fit Points” analysis of the data produced an estimate of 3,733,000 copies as the initial number of cDNA copies in the cellular DNA sample.


LightCycler Data Analysis (muench) - [Quantification: 990126~2; F2; tPA-HP-PCR seg 2; CCC 1-10-25]
File Quantification Report Window Help
Analysis
Fit Points
Second Derivative Maximum
Baseline Adjustment
None
Arithmetic
Proportional
Normalized
Number of Points
2
Show Fit Points
P... Name Standard Calculated... Cro...
1 W 10E8 131,800,000 11.09
2 W 10E7 23,630,000 13.73
3 W 10E6 1,847,000 17.63
4 W 10E5 139,100 21.59
5 W 10E4 30,940 23.90
6 Wn10E3 2,387 27.82
7 H20 1,960 28.12
8 W 10E8 +C 10E4 100,000,000 88,270,000 11.71
9 W 10E7 +C 10E4 10,000,000 10,770,000 14.93
10 W 10E6 +C 10E4 1,000,000 755,000 19.00
11 W 10E5 +C 10E4 100,000 129,400 21.70
12 W 10E4 +C 10E4 10,000 10,320 25.58
13 W 10E3 +C 10E4 1,000 921.3 29.28
14 H20 +C 10E4 1.000 1.270 39.37
15 2.2 x 10E4 Zellen + C 3,733,000 16.55
Step 1: Baseline
Step 2: Noise Band
Step 3: Analysis
Fluorescence (F2)
Cycle Number
Linear Regression
Crossing Points
Slope = -3.527
Intercept = 39.73
Error = 0.372
r = -1.00
Cycle Number
Log Concentration

6. Rapid Genotyping and Mutation Analysis with the LightCycler

6.1 Basic Information about Genotyping and Mutation Analysis

Why Detect Mutations?

Driven largely by such programs as the Human Genome Project, molecular genetics has developed new tools for rapid detection of novel genes. As a result, there has been an exponential increase in the number of newly discovered genes.
Many of these newly discovered genes are likely to be involved in pathogenic processes. Alterations of gene sequences (mutations) have an especially detrimental effect on normal cell development.
However, linking altered gene expression to a particular disease phenotype is an enormous job. It is clear that a rapid and simple mutation detection method is essential to exploration of the cellular function of individual genes.

Limitations of Conventional Techniques for Mutation Analysis

Methods for detecting mutations fall into two general types:

- Mutation screening techniques, to detect novel mutations, and
- Parameter-specific methods, to detect mutations of known phenotype.

Several approaches have been described and reviewed (Dianzani et al. 1993; Cotton 1997). However, only a few of these methods meet all these requirements of an effective detection method:

- High specificity and sensitivity
- Low costs
- Speed
- Ease of use
- Avoidance of hazardous reagents

Note: Some of these methods, including SSCP (Single-Strand Conformation Polymorphism), DGGE (Denaturing Gradient Gel Electrophoresis) and Heteroduplex Analysis, are not rapid, since they require time-consuming electrophoretic separation. Other methods, such as RFLP (Restriction Fragment Length Polymorphism) and EMC (Enzyme Mismatch Cleavage) are complex, requiring multiple enzymatic reactions. Still others, e.g. sequencing, are too expensive for routine use.

Continued on next page

6.1 Basic Information about Genotyping and Mutation Analysis, Continued

Mutation Analysis with the LightCycler

Most mutation detection systems have three stages:

1. Amplification (e.g., PCR),
2. Discrimination (e.g., restriction digest), and
3. Detection (radioactive or non-radioactive).

In conventional methods, each of these stages requires extensive manipulation of the samples. In contrast, in the LightCycler, the only manipulation necessary is initial setup of the PCR. Amplification, discrimination, and detection are automatically performed in the same sealed reaction vessel. Thus, the LightCycler offers a method for detection of mutations that is more reliable, rapid, and convenient than conventional detection methods.

Note: For examples of mutation detection with the LightCycler Dual Color Detection method, see Section 5.3 of this chapter.

6.2 Mutation Analysis with the LightCycler: Single Color Detection Method

Introduction

The LightCycler Hybridization Probes detection format offers a conceptually novel approach for genotyping and/or detection of mutations:

1. The physical parameter used to discriminate between two amplicons (e.g., wild type and mutant) is the melting temperature (T_m)of sequence-specific hybridization probes.
2. Detection is performed online.
3. Since the target can be amplified and then melted rapidly in a small volume, results can be obtained in <45 minutes.

Principle

When a sample is heated, sequence-specific hybridization probes melt off the target sequence at a characteristic temperature (T_m of the probes). This melting can be monitored by changes in sample fluorescence. (see "Sequence-Specific Detection with Hybridization Probes", Section 1.3.2 of Chapter C.) A single base mismatch within the region complementary to the probe will greatly decrease the probe's stability and, thus, lower its melting temperature. The technique can distinguish wild type, mutant, and heterozygous mixes of the two by differences in the T_m at which the probes melt off each type of gene.

Single Color Detection

To perform a single color detection / genotyping experiment with the LightCycler, set up the instrument in Hybridization Probes detection format (see, for instance, Sections 2.2 and 5.3 of this chapter).
In brief, the reaction mix will contain template, specific primers, the LightCycler - DNA Master Hybridization Probes (which contains PCR enzymes and buffers), and a pair of fluorescently labeled, sequence-specific hybridization probes.
One of the hybridization probes, the **mutation probe**, will be complementary to the suspected site of the mutation. The adjacent probe, the **anchor probe**, binds more tightly to the target DNA than the mutation probe. This ensures that only the T_m of the mutation probe is measured during melting point analysis.

Continued on next page

6.2 Mutation Analysis with the LightCycler: Single Color Detection Method, Continued

Design of Mutation Probe

Follow these guidelines to design a successful mutation probe:

- The mutation probe should be located over the mutation site in such a way that the mutation lies in the center of the region recognized by the probe.
- The T_m of the mutation probe should be 5°–10°C higher than the T_m of the primers.
- In general, the length of the probe should be 15–30 nucleotides.
- The probe should be free of secondary structures.

Note: For more information on hybridization probe design, see Section 1.3.2.1 of Chapter C.

Design of Anchor Probe

Follow these guidelines to design a successful anchor probe:

- The sequence recognized by the anchor probe must be adjacent to that recognized by the mutation probe. When the two probes are bound to target, the gap between them should be only 1–5 nucleotides.
- The T_m of the anchor probe should be 5°–10°C higher than the T_m of the mutation probe.
- The probe should be free of secondary structures.

Note: For more information on hybridization probe design, see Section 1.3.2.1 of Chapter C.

Main Steps of the Single Color Detection Procedure

Mutation analysis with the LightCycler Single Color Detection method has the following stages:

Stage	Description
1	The target sequence is amplified in the LightCycler by standard PCR.
2	After amplification, a melting curve analysis is performed. In brief, during the analysis the temperature is gradually (0.2°C) increased from below primer annealing temperature to above denaturing temperature. The fluorescent signal is continuously monitored during this slow ramping.
3	The mutation probe from a hybridization probe pair will melt off the target DNA at a characteristic T_m, producing a rapid drop in the fluorescence signal. ***Note:*** A mismatch between probe and target sequence, e.g., caused by a mutation, will destabilize the probe-target duplex. This lowers the T_m. In general, a single base mismatch will lower the T_m by 3°–10°C, which is sufficient for reliable discrimination.

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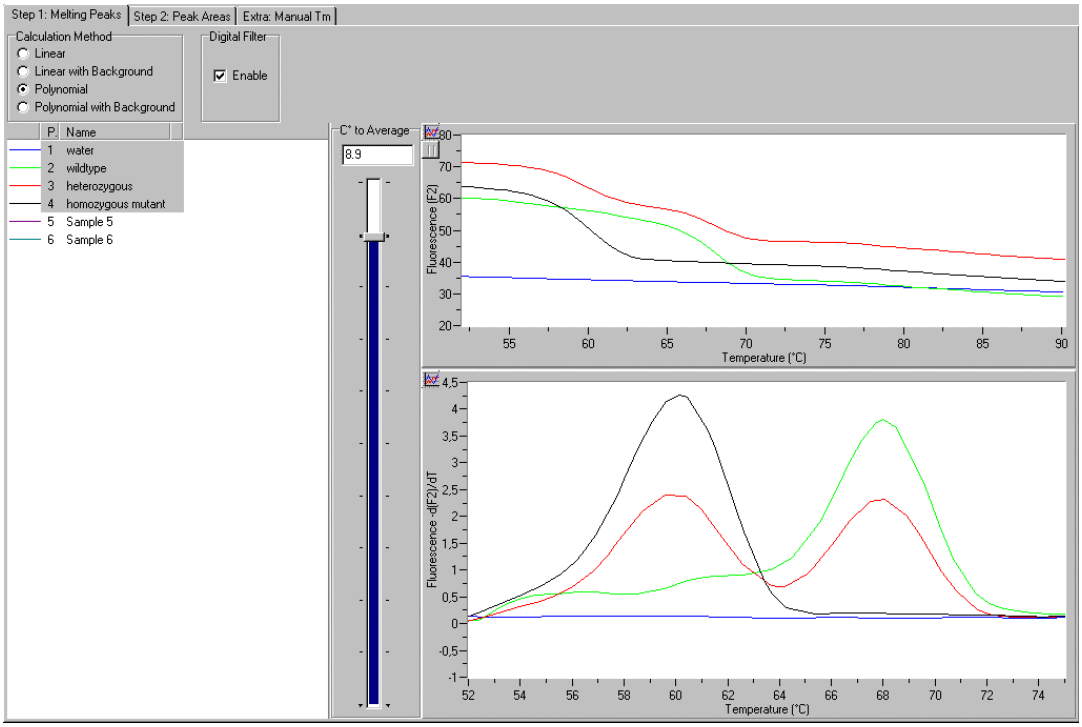
6.2 Mutation Analysis with the LightCycler: Single Color Detection Method, Continued

Typical Result: Single Color Detection of a Mutation

The picture below shows the detection of a single base-pair (missense) mutation with the LightCycler. DNA from homozygous (wild type and mutant) and heterozygous genotypes were amplified and analyzed with the LightCycler in the presence of sequence-specific hybridization probes. The different melting behavior of each sample is shown in the upper plot. The first negative derivative (-dF/dT) of these melting curves (lower plot) is used to determine the melting temperature (T_m).



Result: The T_m of each sample allows discrimination of the different genotypes. Heterozygotes show composite melting peaks, which represent signals obtained from both the mutant and wild type allele.

7. Troubleshooting

7.1 Troubleshooting LightCycler PCR

Introduction

This section describes various problems that may occur during a LightCycler run and recommendations for avoiding or correcting them.

Correcting Sample Problems

To avoid or correct various problems that may affect LightCycler samples, follow the recommendations in the table below.

Problem	Possible Cause	Recommendation
LC does not find any samples to analyze	There is no dye in the reaction mixture	Do not use H_2O alone as a negative control. Instead, combine "master" reaction mix, which contains the dye, with H_2O (in place of nucleic acid sample) to make the control.
	The dye has been exposed to light and bleached	• Do not expose the master reaction mix for SYBR Green or Hybridization Probes to light • Do not freeze or thaw the master mix repeatedly.
	• The PCR reaction mix is still in the upper capillary reservoir, or • An air bubble is trapped in the glass capillary tip	Repeat capillary centrifugation.
	Temperature used for the Seek Process (see Chapter B) for SYBR Green I samples is too high (e.g., 95°C). Thus, no double stranded DNA remains to bind the dye and produce fluorescent signal	• Check temperature window on the Loading Screen. • Use 30°C for PCR, and 50°-55°C for RT-PCR.
	RNA preparation contains a high concentration of guanidinium chloride	Purify RNA template with the High Pure RNA Isolation Kit.

Continued on next page

7.1 Troubleshooting LightCycler PCR, Continued

Correcting Sample Problems **(continued)**

Problem	Possible Cause	Recommendation
The LightCycler does not find the last sample capillary in the carousel	User has left gaps (empty holes) between sets of samples during insertion of the capillaries into the rotor	Set the value "Number of samples" to include gaps in the sample order, since the value tells the machine the last rotor position to be analyzed (see Chapter B). ***Note:*** The LightCycler will identify gaps in the sample order during the Seek Process and will notify user about them.
The names of the samples are not displayed on the Run Screen	• Sample names were not entered into the Loading Screen, or • Samples were named during the Seek Process	Always enter sample names into the Loading Screen • Prior to the run, or • During the run. • Do not enter sample names during the Seek Process; such names will be rejected
The instrument does not respond when the **Run** button is pressed, and no heating occurs	The LightCycler was not turned on before user started to program the run	Leave the Run Mode by either pressing the **Exit** button or using the task manager to close the task. Start over by programming the run again.
	Lid is not closed properly	Re-insert the sample carousel and close the lid completely.
Log-linear phase of amplification just starts when the amplification program finishes	Starting concentration of nucleic acid is very low	• Optimize PCR conditions ($MgCl_2$ concentration, primer and probe design), • Increase amount of starting material, and • Repeat the run.
	Too few cycles in program	• Use the "Add 10 Cycles" button to increase number of cycles in the cycle program.

Continued on next page

7.1 Troubleshooting LightCycler PCR, Continued

Correcting Sample Problems **(continued)**

Problem	Possible Cause	Recommendation
No amplification occurs	Wrong channel used to monitor amplification online	In the Programming Screen, change the monitoring channel in the Display Mode field. ***Note:*** Don't worry, the data will be recorded and saved.
	Pipetting errors or omitted reagents	Check all reagents, especially: • Titrate $MgCl_2$ concentration. • Check for missing or defective dye.
	Chosen gain settings are too low	Use the Real Time Fluorimeter option to optimize gain settings. Change gain settings in the cycle programs to the optimal values and repeat the run.
No amplification occurs (continued)	Graph set to wrong scale	To select a better scale for the x- and y-axis, adjust the scaling of these axes as described in Chapter B of this manual.
	Measurements do not occur	Check the cycle programs. Choose "Single" acquisition mode at the end of the following stages: • Annealing (for Hybridization Probes format), or • Elongation (for SYBR Green I detection).
	Amplicon length is > 1 kb	Do not use amplicons > 1 kb. ***Note:*** Amplification efficiency is reduced with large amplicons.
	Impure sample contains PCR inhibitors	• Do not use more than 8–10 µl of DNA per 20 µl PCR reaction mixture. • Repurify the nucleic acids to remove inhibitory agents.
	High GC-content of primers and hybridization probes	• Add DMSO (2-10%) to the reaction mixture. • Redesign primers and probes.
	Unsuitable hybridization probes	Check sequence and target location of hybridization probes.
	No positive control	Always include a positive control.

Continued on next page

7.1 Troubleshooting LightCycler PCR, Continued

Correcting Sample Problems **(continued)**

Problem	Possible Cause	Recommendation
Fluorescence intensity is too low	Dye concentrations in reaction mix are too low or dyes have deteriorated because the master mixes were improperly stored	• Store all dye-containing reagents at –20 °C. Protect them from light. • Do not freeze and thaw the reagents repeatedly. • To improve low signals from Hybridization Probes, make the concentration of the LC Red-labeled probe twice as high as that of the fluorescein-labeled one.
	Poor PCR efficiency due to suboptimal reaction conditions	• Titrate $MgCl_2$ concentration. • Adjust the primer and probe concentrations to 0.3–1.0 µM. • Check annealing temperature of primers and probes. • Check experimental protocol. • Always run a positive control along with your samples.
	During mutation analysis with hybridization probes, the melting temperature of the hybrid formed between the mismatch strand and the hybridization probe is lower than the annealing temperature of the primers. Thus, the hybridization probes can't bind and create a signal.	Make sure the melting curve analysis starts at a temperature below the PCR annealing temperature. Then you will get a clear signal and will be able to interpret the data. ***Note:*** This will not affect amplification efficiency.
Signals from all samples show periodic variations	O-ring on top of sample carousel is not in place and thus can't immobilize capillaries properly	Re-insert O-ring in the top of the sample carousel.
Negative control samples show a significant positive (i.e., above background) signal	For SYBR Green I format: • Contamination, or • Primer-dimer formation For Hybridization Probes format: • Contamination	• Remake all critical solutions. • Pipette samples in a clean, dedicated work area. • Close lid of the negative control reaction tube immediately after pipetting. • Use heat-labile UNG to prevent carry-over contamination (see Section 2.3.5).

Continued on next page

7.1 Troubleshooting LightCycler PCR, Continued

Correcting Sample Problems **(continued)**

Problem	Possible Cause	Recommendation
Signals are too high and reach overflow	Unsuitable gain settings	Gain settings cannot be changed during or after a run! Before repeating the run: • Check that the LED is set on **Calibrated**, • Use the Real Time Fluorimeter option to find suitable gain settings. ***Caution:*** At measuring temperature, the background fluorescence should be <10 for SYBR Green I and <20 for Hybridization Probes. ***Note:*** Use an extra sample for this procedure to avoid bleaching of dyes.
High background in Hybridization Probe format	Hybridization signals are very low, thus the background seems to be relatively high	Follow general optimization strategies for LightCycler PCR (see Section 2.4).
	Hybridization probe concentration is too high	Hybridization probe concentration should be 0.3–1.0 µM.
	Poor quality of hybridization probes	Order a new pair of hybridization probes.
	Gain settings are too high	Reduce value of gain setting. ***Note:*** Use the Real Time Fluorimeter option to optimize the gain settings.
In late cycles, amplification curve decreases after reaching a plateau (Hybridization Probes format)	"Hook effect" (competition between binding of the hybridization probes pair and re-annealing of the PCR product)	To avoid "hook effect", perform an asymmetric PCR; favor the amplification of the DNA strand recognized by the hybridization probes. ***Note:*** The effect does not affect interpretation of the results.

7.1 Troubleshooting LightCycler PCR, Continued

Correcting Problems with Melting Curve Analysis

To avoid or correct various problems that may affect Melting Curve Analysis, follow the recommendations in the table below.

Problem	Possible Cause	Recommendation
Low fluorescence intensity after amplification	Gain settings are unsuitable	Use the Real Time Fluorimeter option to optimize gain settings. ***Note:*** Even weak amplification signals are sufficient for interpretation of melting curve results.
Melting peak is very broad and peaks can't be differentiated	**Points to Average** setting is too high	• Lower the number **of Points to Average.**

Double peak appears for one product	• Two products of different length or GC content have been amplified
	• Pseudogenes
	• Mispriming
		• Check products on an agarose gel.
		• Increase hybridization stringency by doing the following:
		  • Redesign primers.
		  • Check annealing temperature.
		  • Perform a "touch-down" PCR.
		  • Use hybridization probes for better specificity.

Melting temperature of a product varies from experiment to experiment	Variations in reaction mixture, especially salt concentration	• Check purity of DNA/RNA template. • Reduce variability in parameters such as $MgCl_2$, heat-labile UNG, Hot Start antibody, or program settings.

Continued on next page

7.1 Troubleshooting LightCycler PCR, Continued

Correcting Problems with Melting Curve Analysis **(continued)**

Problem	Possible Cause	Recommendation
• No specific PCR product peak appears; only peak is due to a primer-dimer • Very large primer-dimer peak	Amplification of primer-dimers has out-competed amplification of specific PCR product	• Keep all samples at 4°C until the run is started. • Keep time between preparing the reaction mixture and starting the run as short as possible. • Increase starting concentration of DNA template. • Titrate $MgCl_2$. • Increase annealing temperature to enhance stringency. • Redesign primers. • Use Hot Start antibody (see Section 2.3.6).
Primer-dimer and product peaks are very close together	Primers have unusually high GC-content	• Redesign primers. • Run melting curve with low ramping rate (0.1°C/sec, continuous measurement). • Expand scale of x-axis. • Decrease "**°C to Average**" value.
Very broad primer-dimer peak with multiple peaks	Primers are not homogeneous, which leads to primer-dimer variations (concatemers, loops, etc.)	• Redesign primers. • Use Hot Start (see Section 2.3.6)
Products detected with hybridization probes have no precise melting peak	• Hybridization probes are not homogeneous and/or contain secondary structures • Pseudogenes lead to multiple PCR products	• Redesign hybridization probes. • Check PCR products on an agarose gel.
All samples contain single peak and peak is identical in all samples.	All samples contaminated	• Close capillaries during centrifugation step. • Use fresh solutions.

7.1 Troubleshooting LightCycler PCR, Continued

Correcting Problems in Quantification

To avoid or correct various problems that may affect LightCycler quantification, follow the recommendations in the table below.

Problem	Possible Cause	Recommendation
Amplification curves have very flat slopes	Poor PCR efficiency	Optimize PCR as follows: • Titrate $MgCl_2$ concentration. • Increase quality of primers and hybridization probes. • Increase purity of template
Fluorescence signals vary	Pipetting errors	• To minimize pipetting errors, set the instrument to analyze data in the F2/F1 or F3/F1 mode (not suited for dual color applications). • Use option "Proportional" for baseline adjustment.
	Variations in capillary position	Check proper position of each capillary in the rotor. ***Note:*** Push all capillaries down into the rotor hole until the first rim of the plastic reservoir is level with the top of the sample hole.
	O-ring on top of the carousel is not in place and thus can't immobilize capillaries properly	Remove O-ring and re-insert it in the top part of the carousel.
	Signal intensity very weak, thus amplifying the effect of minor signal variations	Optimize PCR conditions.
	Skin oils have been deposited on the surface of the capillary tip	Always wear gloves when handling the capillaries.

Continued on next page

7.1 Troubleshooting LightCycler PCR, Continued

Correcting Problems in Quantification **(continued)**

Problem	Possible Cause	Recommendation
SYBR Green I format: Amplification curve of one sample reaches plateau at a lower signal level than the other samples	• Starting amount of genomic DNA is too high; DNA binds too much dye and leads to a high background signal. Remaining amounts of unbound dye are insufficient to monitor product increase during amplification. • Dye has bleached	• Do not use more than 50 ng of complex genomic DNA (PCR) or 100 ng of total RNA (RT-PCR) in a 20 µl reaction volume. • Use Hybridization Probe format, which allows sample to be increased to 500 ng DNA or 1 µg total RNA. • Make sure reagents containing the dye have been stored protected from light. • Do not repeatedly freeze and thaw reagents that contain dye.
The standard curve, as well as the calculated concentration data, are not displayed	During sample input, the standards have not been labeled **Standard** and/or the concentration value of the standards is missing	• Always be sure to label external standards as Standards in the Loading Screen, and **specify the concentration of each standard**. • Repeat the run, and check the parameters in the Loading Screen.
	Values of samples selected for the standard curve are below noise band level	Deselect samples that do not have signals above noise band level.
Standard curve is unrealistic (red line looks "zigzag")	Standards were placed into the sample carousel in a different order than that specified in the Loading Screen	Repeat the experiment and carefully check that sample order in the rotor is the same as in the Loading Screen.

7.1 Troubleshooting LightCycler PCR, Continued

Correcting Problems in RT-PCR

To avoid or correct various problems that may affect LightCycler RT-PCR runs, follow the recommendations in the table below.

Problem	Possible Cause	Recommendation
Low RT-PCR signal intensity	Temperature for reverse transcription reaction too high	Use 55°C in a one-step RT-PCR. ***Caution:*** Using 60°C will reduce the cDNA yield drastically.
	RNase contamination	• Use only RNase-free water and solutions. • Check integrity of RNA. • Store RNA at –70 °C. • Wear gloves.

	RNA solution contain RT-PCR inhibitors	• Purify RNA to remove:
		  • Guanidinium chloride (reverse transcription inhibitor), or
		  • PCR inhibitors (e.g., bilirubin, heparin, ethanol, etc.)

	Reverse transcriptase denatured prior to reverse transcription	The reverse transcriptase is temperature sensitive and will be inactivated at high temperatures. Change cycle program to include a lower temperature reverse transcription step.
	$MgCl_2$ concentration not optimized	Titrate $MgCl_2$ concentration for each new system. Try runs with several $MgCl_2$ concentrations between 3 and 8 mM, then choose the one that gives the best result.
	RNA concentration too high or too low	Titrate amount of template RNA; try runs with 1 – 500 pg template.
	cDNA concentration too high or too low (two-step RT-PCR)	Titrate amount of cDNA.

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7.1 Troubleshooting LightCycler PCR, Continued

Correcting Problems in RT-PCR **(continued)**

Problem	Possible Cause	Recommendation
Additional peaks in the melting curve after RT-PCR	Temperature for reverse transcription too low	Do not use a temperature <55°C for reverse transcription. ***Note:*** Temperatures <55°C favor nonspecific PCR products.
	RNA contaminated with DNA	• Check RNA quality. • Use mRNA. • Perform a control reaction containing only RNA and the. LightCycler DNA Master (to check for presence of DNA).
	Temperature was too low during Seek Process	Conduct the Seek Process at a higher temperature by entering the appropriate temperature in the field at the left corner of the Loading Screen.
Amplification curve appears to be bent during the exponential phase	Primer problem	• Redesign primers. • Repurify primers.
Melting peak of negative control shows a higher T_m than specific PCR product	DNA contamination in control sample	• Check water for RNA and DNA contamination. • Repeat the experiment using fresh solutions.

Chapter E:

Appendix

1. Appendix

1.1 Table of contents

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2. Technical Support

Technical Support

For Technical Support, please contact your local Roche Molecular Biochemicals' representative. Please have your serial number available when contacting us.

Person to contact	
Phone number	
FAX number	
E-mail address	

2.1 Warranty

Warranty

Warranty conditions are specified in your purchase contract. Please contact your Roche Molecular Biochemicals representative for detailed information.

2.2 Maintenance

Maintenance

Generally, the LightCycler is maintenance free.

3. Ordering List

Hardware

Product	Pack Size		Cat. No.
LightCycler Instrument	1 1 96 32 1 1 1 1 1 1 1	LightCycler Instrument Sample Carousel, mounted in the LightCycler Capillaries and caps in a box Adapters, 1 cooling box for adapters LightCycler Operator's Manual (DIN A4-binder) Computer with monitor Software Package Cable to connect LightCycler instrument with the computer Power cord (German) Power cord (US) Mouse pad	2 011 468
LightCycler Capillaries	8x 8x	96 Capillaries 96 Stoppers	1 909 339
LightCycler Sample Carousel	1	Rotor for 32 capillaries	1 909 282
LightCycler Adapters	1x	32 Microcentrifuge adapters	1 909 312
LightCycler Software Package	1x	CD, Operator's Manual, mouse pad	1 909 304

Generic Kits

Product	Pack Size	Cat. No.
LightCycler™-DNA Master SYBR® Green I	1 kit (96 reactions)	2 015 099
LightCycler™-DNA Master Hybridization Probes	1 kit (96 reactions)	2 015 102
LightCycler™-Control Kit DNA	1 kit (50 reactions)	2 015 129
LightCycler-RNA Amplification Kit SYBR Green I	1 kit (96 reactions)	2 015 137
LightCycler-RNA Amplification Kit Hybridization Probes	1 kit (96 reactions)	2 015 145
LightCycler-Control Kit RNA	1 kit (50 reactions)	2 015 153

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3. Ordering List, Continued

Labeling Reagents

Product	Pack Size	Cat. No.
LightCycler-Red 640-NHS Ester	For labeling of 5x 50 nmol oligonucleotide	2 015 161
LightCycler-Red 705-Phosphoramidite	For labeling of 10 oligonucleotides (0.2 µmol scale)	2 157 594

Reagents

Product	Pack Size	Cat. No.
Uracil DNA Glycosylase, heat-labile	100 units 500 units	1 775 367 1 775 375

4. Further Reading

LightCycler™ Technology

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Melting Curve Analysis

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4. Further Reading, Continued

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PCR in general

- Thein SL, Wallace RB (1986): The use of synthetic oligonucleotides as specific hybridization probes in the diagnosis of genetic disorders. In: Human Genetic Diseases: A practical approach. Ed.: Davis KE. Herndon, VA: IRL Press

SYBR Green I

- Ames BN, Durston WE, Yamasaki E & Lee FD (1973): Carcinogens are Mutagens: A Simple Test System Combining Liver Homogenates for Activation and Bacteria for Detection. *Proc. Natl. Acad. Sci. USA* **70**: 2281-2285
- Maron DM & Ames BN (1983): Revised methods for the Salmonella mutagenicity test. *Mutation Research* **113**: 173-215